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Abstracts

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MOTOR AND FUNCTIONAL CONTROL OF SKELETAL MUSCLE

C-TERMINAL TRUNCATION OF CARDIAC TROPONIN I IMPAIRS KINETICS OF FORCE RELAXATION IN SKELETAL AND CARDIAC SINGLE MYOFIBRILS.

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C-terminal proteolysis of cardiac troponin I (cTnI) is thought to play a important role in cardiac ischemia, including myocardial stunning. We directly investigated the mechanical consequences of cTnI degradation in single myofibrils. Full length cTnI ($cTnI_{FL}$) or truncated cTnI ($cTnI_{1-192}$) were expressed in *E.coli*, purified, reconstituted with other cardiac troponin (Tn) subunits and subsequently exchanged into single myofibrils. Passive and active contractile properties were measured in rabbit psoas myofibrils in which native Tn complex had been replaced by a cardiac Tn containing either the $cTnI_{FL}$ or the $cTnI_{1-192}$. Myofibrils were mounted in a force recording apparatus and calcium activated by rapid solution switching. The presence of $cTnI_{1-192}$ in the cTn complex decreased maximal force (P_0), the rate of force activation (k_{ACT}) and the rate of force redevelopment (k_{TR}) by about 25%. In $cTnI_{1-192}$ containing myofibrils the kinetics of force relaxation were markedly prolonged whereas calcium sensitivity was increased as compared to $cTnI_{FL}$ containing myofibrils. The passive tension-sarcomere length relationship was also altered by the presence of $cTnI_{1-192}$ in the cTn. The modifications seen in passive tension indicated that $cTnI_{1-192}$ may be unable to fully inhibit acto-myosin interaction in the absence of Ca^{2+}. A flawed inhibition of acto-myosin interaction may explain the marked reduction of P_0 found in $cTnI_{1-192}$ containing myofibrils. Similar preliminary experiments in human cardiac ventricular myofibrils confirmed a marked slowing down of the kinetics of relaxation in $cTnI_{1-192}$ containing myofibrils. These results suggest that C-terminal cTnI proteolysis may alter cardiac mechanics mainly by affecting the diastolic function.

CHRONIC ATRIAL FIBRILLATION IMPAIRS FORCE GENRATION OF HUMAN ATRIAL SINGLE MYOFIBRILS.

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Chronic atrial fibrillation (cAF), the most common sustained arrhythmia, is accompanied by atrial contractile dysfunction, which may persist for up to 1 month after cardioversion. The contractile dysfunction can only be partly attributable to impaired calcium handling and myocyte myolisis and may be due to alterations of the contractile apparatus itself. However it has never been directly studied at the sarcomere level. We used single human atrial myofibrils and rapid solution switching to investigate the changes in active and passive tension associated to cAF and the possible associated changes in isoform expression pattern of two sarcomeric proteins: myosin heavy chain (MHC) and titin. Systolic and diastolic contractile properties were measured in myofibrils prepared from the right atrial appendage of 12 cAF patients and 10 patients with sinus rhythm (SR) undergoing open heart surgery. In cAF diastolic tension was depressed and maximum systolic force (90±7 mN mm^{-2}) showed a 30% reduction whereas Ca^{2+}-sensitivity was increased. In cAF, the rate of Ca^{2+} force activation (k_{ACT} 2.0±0.1 s^{-1}), the rate constants of relaxation phase (slow k_{REL} 0.4±0.04 s^{-1}; fast k_{REL} 10.0±0.7 s^{-1}) were markedly slower than in SR (k_{ACT} 3.73±0.18 s^{-1}, slow k_{REL} 0.52±0.04 s^{-1}; fast k_{REL} 16.0±1.0 s^{-1}). The slower kinetics seen in cAF could be partly explained by a shift of MHC isoform expression (from α-MHC to β-MHC). The 2 titin isoforms N2B and N2BA were co-expressed in cAF and SR atria, but the proportion of N2BA was 22%

higher in cAF. This was consistent with the reduction in diastolic tension seen in cAF. This suggests that the atrial contractile dysfunction accompanying cAF has relevant sarcomeric basis.

ESTIMATION OF HUMAN MUSCLE FORCE DYNAMIC RESPONSE USING A SHORT FEW SECONDS STIMULATION PROTOCOL.

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Aim of the study was to compare the dynamic responses of the tibialis anterior (TA) muscle-joint unit obtained after stimulation with:

a) 14 separated sinusoidal inputs;
b) a short few seconds stimulation containing 7 sines.

Ten subjects (age: 23-50) volunteered for the study. The leg was fixed in a dynamometer to detect the torque generated during isometric contractions of the ankle flexors. The amplitude of a 30 Hz stimulation train administered at the TA motor point was varied sinusoidally, thus changing the number of the recruited motor units, and hence the tension at the tendon in the same fashion. During 5.18 s the muscle was stimulated with a sequence of different sinusoidal frequencies (0.4, 1.0, 1.8, 2.5, 3.0, 4.5, 6.0 Hz; one period per frequency) termed as “sweep”. After the sweep a sequence of 14 isolated frequencies (0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 Hz), termed “steps”, was administered. Each step lasted 5 s with a resting period of 3 min between one and the following: the total duration of the protocol was ~ 40 min.

For both protocols, a and b, the Bode plots for average (mean ± SD) gain attenuation and phase shift, reporting the amplitude reduction and phase shift with respect to the input sine, were calculated.

From the Bode plots it was possible to model the force dynamic response of TA by a critically damped II order system with two real coincident poles + a pure time delay:

a) for the 14 steps: poles at 2.04 Hz and pure time delay of 15.6 ms;

b) for the sweep: poles at 2.19 Hz and pure time delay of 13.2 ms.

The similarity of the steps and sweep transfer functions, obtained from the two stimulation protocols, suggests that it is possible to characterize the in-vivo mechanics of muscle-joint unit with a short (few seconds) sinusoidal stimulation. The frequency response could be used to design computerized controllers for rehabilitation devices and develop models of human movement for occupational and sports activities.

MECHANICAL AND STRUCTURAL PROPERTIES OF FORCE GENERATORS IN SKELETAL MUSCLE: EFFECTS OF SOLUTION TONICITY.

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In skeletal muscle fibres isometric tension decreases and increases with hypertonic and hypotonic solution respectively. In the present work we concentrated on effect of tonicity on force and extension of crossbridges with a combined mechanical and ultrastructural X ray diffraction study.
Fast stretches were applied to intact frog muscle fibres (*Rana esculenta*) at tetanus plateau and during tetanus rise in normal Ringer, hypertonic (1.4T) and hypotonic (0.8T) solution at 14°C. In the same experimental conditions, synchrotron radiation was used to measure the intensity of *M3* reflection (I_{M3}) during sinusoidal length oscillations.

During fast stretching tension rises steeply to a peak (critical tension, P_c) and then falls to a much lower lever because of forced crossbridges detachment. During the tetanus rise P_c is directly proportional to tension developed, providing a valid method to estimate the number of attached crossbridges. The ratio of P_c over isometric tension and the sarcomere elongation needed to cause force crossbridge detachment (L_c) increases and decreases in hypertonic and hypotonic solution respectively. I_{M3} signals are approximately sinusoidal and in phase opposition to fibre length and force but are distorted during shortening by the passage of S1 myosin through a maximum intensity position. Hypotonicity increased I_{M3} distortion and hypertonicity decreased it. In conclusion, tension variations with tonicity are not caused by a change of attached crossbridges number but by a different force per single crossbridge. As showed by the different L_c and I_{M3} distortion values, the myosin head mean position depends on tonicity solution shifting towards the maximum intensity position by lowering the tonicity.

SPACING AND INTENSITY CHANGES IN THE MYOSIN-BASED X-RAY REFLECTIONS DURING ISOMETRIC FORCE DEVELOPMENT.

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Isometric force development in skeletal muscle is accompanied by an increase in the spacings of the third and sixth order myosin-based meridional reflections: the M3 and M6 spacings (S_{M3} and S_{M6}) are 14.34 nm and 7.16 nm respectively at rest and 14.57 nm and 7.29 nm at the tetanus plateau (T_0) (Linari *et al.*, *PNAS USA*, 97:7226, 2000). These changes are one order of magnitude larger than those expected from the instantaneous elasticity of the myosin filament (Reconditi *et al.*, *Nature* 428:578, 2004). We recorded X-ray diffraction patterns from intact skeletal muscle fibres of *Rana temporaria* (2.1 µm sarcomere length, 4°C) with 5 ms time resolution during isometric force development and redevelopment following unloaded shortening of 100 nm per half-sarcomere. The ~1.5% increase in S_{M6} during force rise leads that of S_{M3} by about 10 ms. Unloaded shortening imposed 5 ms after the start of stimulation to delay force rise transiently reduces S_{M3} and only delays the increase of S_{M6}. During unloaded shortening imposed at the tetanus plateau S_{M3} drops progressively to its resting value, while S_{M6} drops mid-way between its plateau and resting values. The data are interpreted assuming that the M3 reflection originates mainly from the myosin heads and records the time course of head attachment and force generation, while the M6 reflection originates from periodic structures on the backbone of the thick filament. Supported by MIUR (Italy), MRC (UK), NIH (USA), ESRF, EMBL.

THE INTERFILAMENTARY DISTANCE OF INTACT FIBRES FROM FROG MUSCLE IN DIFFERENT PHYSIOLOGICAL STATES.

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In an intact fibre from frog muscle at sarcomere length ~2.2 µm the interfilamentary spacing, estimated with X-ray diffraction as the distance between the lattice planes made by the myosin filaments ($d_{1,0}$, ~34 nm) hardly changes upon activation and tension development (Bagni et al., *Biophys. J.* 67:1965, 1994). However, in a fibre swollen by the skinning, $d_{1,0}$ is reduced from 42 nm to 39 nm upon activation (Matsubara & Elliott, *J. Mol. Biol.* 72:657, 1972) or induction of rigor (Brenner & Yu, *J. Physiol.* 441:703, 1991; Kawai et al., *Biophys. J.* 64:187, 1993), suggesting that myosin head attachment to actin implies a radial force that tends to reverse the increased interfilamentary distance. Here we reinvestigate the problem by using intact fibres from the frog (sarcomere length 2.2 µm, 4 °C) in which the rigor state is induced by metabolic poisons. Before rigorisation $d_{1,0}$ was 33.2±1.5 nm (mean±SD, 4 fibres) in the

resting fibers and 33.2±1.6 nm during the isometric contraction. After induction of rigor $d_{1,0}$ increased to 39.5±1.6 nm (+19%), without a significant change in fibre width (+2±6%). Rising the static tension by an amount similar to the isometric tetanic force by slowly stretching the rigor fibre increased $d_{1,0}$ by only 2.8±1.8%, notwithstanding the substantial change in tilt of myosin heads (Reconditi et al., *Biophys. J.* 85:1098, 2003). These results indicate that the interfilamentary distance induced by head attachment is independent of the strain and conformation of the myosin heads and is likely determined by the combination of the steric constraints imposed by the dimension of the attached heads and the flexural rigidity of the filaments. Supported by MIUR (Italy), MRC (UK), NIH (USA), ESRF, EMBL.

KINETICS OF MYOSIN-ACTIN INTERACTION STUDIED WITH ISOTONIC VELOCITY TRANSIENTS IN SKINNED MUSCLE FIBRES FROM RABBIT PSOAS.

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Shortening in muscle contraction is due to a working stroke in the globular head of the motor protein myosin II that drives the actin filament toward the centre of the myosin filament. The working stroke is associated to the release of the products of the ATP hydrolysis, mainly inorganic phosphate (Pi). A stepwise drop in force (complete in 120 µs) from the steady value attained during the isometric contraction (T_0) elicits an isotonic velocity transient that allows to define the mechanical and kinetic properties of the molecular motor myosin II *in situ* (Piazzesi et al., *J Physiol* **545**:145, 2002). We investigated how the inorganic phosphate (Pi) influences the isotonic velocity transient elicited by force steps to 0.5 T_0 in Ca^{2+} activated skinned fibres from rabbit psoas (2.5 µm sarcomere length, 12 °C). In control conditions ([Pi] ~1 mM) the rate of phase 2 shortening (r_2) was 2.3±1.0 ms^{-1} (mean and SD, 3 fibres); the time after the step taken by phase 3 pause to be complete (t_3) was 18.6±6.0 ms; the speed of phase 4 shortening (V) was 0.3±0.1 µm s^{-1} per half-sarcomere. Increase of [Pi] to 10 mM, reduced T_0 by 34±6%, did not change r_2, reduced t_3 by 40±4% and increased V by 52±17%. The results are discussed in terms of the coupling between Pi release and the myosin working stroke and its regeneration. Supported by NIH (USA, R01 AR049033-03) and MIUR (Italy).

CHANGES IN ELECTRO-MECHANICAL PROPERTIES OF CALF MUSCLES INDUCED BY ACUTE PASSIVE STRETCHING AND TIME COURSE OF RECOVERY IN HUMANS.

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Passive muscle stretching has been shown to diminish maximal force and power output through two main mechanisms: a mechanical alteration of the stretched muscle and an impaired neural activation. The aim of this study was to evaluate i) the stretching-induced changes in the electrical and mechanical properties of the muscle fibres during maximal electrically elicited contractions of the calf muscles; and ii) the time course of these changes after stretching. Seventeen subjects (age 22±1 years, mean±s.e.m.; body mass 75±2 kg; stature 179±2 cm) underwent 3 tetanic electrical stimulation (50 Hz) at maximal intensity, before and after (every 15 min, for 2 hours) a bout of passive stretching. During contractions, surface EMG and mechanomyogram (MMG) were simultaneously recorded from the medial gastrocnemius muscle, together with force. From the analysis of the 3 signals, after stretching it resulted that: i) the force peak, time to peak and the peak rate of force production significantly decreased by 34±6%, 5±2% and 34±6%, respectively; ii) the MMG amplitude (peak-to-peak) also decreased (-14±5%; $p<0.05$); iii) no differences were found in EMG parameters; and iv) maximum force production remained significantly lower even after 2 hours of recovery from the stretching procedures. In conclusion, acute passive stretching affected the mechanical but not the electrical properties of the maximally contracting muscles, suggesting a stretching-induced alteration of the musculotendinous stiffness and contractile capacity, but

not a depression of muscle activation. Moreover, force depression persisted steadfastly from stretching procedures, indicating a recovery time for muscle mechanical characteristics longer than two hours.

HIGHER TRAINING INTENSITY IMPROVES ISCHEMIA-REPERFUSION RESISTANCE IN CHRONICALLY EXERCISED RATS.

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It is well known that regular physical exercise (training) has beneficial effects on the human cardiovascular system. To improve knowledge of the molecular mechanisms underlying exercise-induced cardioprotection in a rat model, 2-month old Sprague-Dawley rats were trained to run on a treadmill using two different protocols at increasing workload (age-matched sedentary animals constituted the control group): MILD (up to ~60% of their VO_{2max}, for 1 h/day, 3 days/week, for 14 weeks) and FULL (80% of their VO_{2max}, 1 h/day, 4 days/week, for 10 weeks). Animals were anesthetized 48 h after the last training session with i.p. sodium thiopental (50 mg/kg) and hearts exposed to 30-min ischemia (closure of the left coronary artery between the pulmonary artery and the left atrium) and 90-min reperfusion. At the end of the treatment, the extent of the risk and infarct areas was determined by colorimetric analysis (Evans blue dying). Whereas the area at risk was unaffected by training (range 37-to-43% of the left ventricle), the infarct area was 49±2%, 38±2% and 28±5% in control, MILD and FULL groups, respectively. Therefore, increasing the load of training improved myocardial protection against ischemia-reperfusion. This preliminary data is presently under consideration in the attempt to correlate the expression of some genes and proteins associated with training-induced cardioprotection.

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TESTING MUSCLE FORCE AND MUSCLE MYOELECTRIC SIGNAL IN FSHD PATIENTS IN A CLINICAL SETTING: DEVELOPMENT OF A NEW PROTOCOL.

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AIM: Devise a protocol to asses neuromuscular functional capacity in patients with facioscapulohumeral muscular dystrophy (FSHD) applicable in a clinical setting. The protocol should provide information on the rate of progression of muscle impairment and discriminate between clinically affected and non affected muscles even in the same subject.

BACKGROUND: Very few quantitative data regarding the neuromuscular performance of FSHD patients are available. Clinical assessment of these patients can be greatly improved by a standardized and reproducible protocol which combines quantitative evaluation of force and neuromuscular output. The proposed protocol includes the concomitant collection of force and surface electromyography (sEMG) data on selected FSHD patients.

METHODS: Elbow flexors maximal voluntary isometric force (MVC) and isometric endurance at 30% and 80% MVC were measured in two patients for both dominant and non dominant side. The sEMG was recorded from the biceps brachii (BB) using 4 electrodes linear arrays in order to measure muscle fibres average conduction velocity (CV).

RESULTS AND DISCUSSION: A previous clinical assessment, indicated that BB muscles were differently affected. The testing results were in agreement with clinical and laboratory data. In both subjects, there was a consistent reduction of MVC and CV on the affected side. Namely CV initial values

were 3 m/s vs 5 m/s and CV decay over time was reduced on the affected side reaching a value of only 2 m/s at the end of the exercise. These results could suggest a fast to slow shift of muscle fibres in the affected muscle.

NON-INVASIVE ASSESSMENT OF MUSCLE FIBRE CONDUCTION VELOCITY DURING AN INCREMENTAL CYCLING TEST.

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Muscle fibre conduction velocity (MFCV) can provide information about motor units recruitment strategies. Recently, MFCV has been measured, non invasively, during constant-load sub-maximal cycling exercise and was found to correlate positively with % of type I myosin heavy chain (Farina et al, 2004,2006).
The present study aimed at verifying the hypothesis that MFCV, measured during an incremental cycling test, could be sensible to the different metabolic requests elicited by the exercise. In particular, the relationship between ventilatory threshold (Tvent), VO_{2max} and MFCV was explored. Five men (28±4 yrs) undertook a one-minute incremental cycling test to exhaustion. Tvent and VO_{2max} were measured using a metabolimeter whilst MFCV with a surface EMG recorded from the vastus lateralis muscle with an adhesive 4-electrodes array.
Reliable measures of MFCV were obtained at every exercise intensity (cross correlation values >0.8). MFCV was positively related to the mechanical work both at VO_{2max} and Tvent occurrence. In particular, a very strong relation (R^2=0.9; P<0.01) was demonstrated between MFCV at Tvent and the corresponding work-load. This indicates that MFCV can be used as non invasive tool to infer motor unit recruitment\derecruitment strategies during dynamic exercise from low to maximal intensities.
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CEREBRAL PLASTICITY IN ELITE ATHLETES: A HIGH-RESOLUTION EEG STUDY

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Electroencephalographic (EEG), electromiographic (EMG), and stabilographic data were simultaneously recorded in elite athletes (11 karate, 8 fencing) and in (8) non-athletes during quiet upright standing at open- and closed-eyes condition. The hypothesis was that when compared to non-athletes, athletes are characterized by enhanced cortical and cortico-muscular information processing as indexed by spectral coherence, directed transfer function (DTF), and event-related desynchronization (ERD) at the dominant rhythms of human brain, namely the alpha rhythms (about 8-12 Hz). Results showed that (i) Stabilographic body sway area did not differ among the groups, and that it was generally lower when visual inputs were available. (ii) Centro-parietal alpha ERD correlated with body sway area in the karate athletes, as a possible effect of cerebral plasticity. (iii) DTF indicated a cortico-muscular over muscular-cortical direction of the coupling of alpha rhythms in both non-athletes and athletes. (iv) EEG-EMG coherence (*gastrocnemius lateralis*) at centro-parietal alpha rhythms was higher during the open than closed eyes condition in the non athletes; this was not true in the karate and fencing athletes, as a

reflection of the optimal cortico-muscular coupling even in the absence of visual stimuli. These results suggest that in elite athletes, the control of quiet upright standing may be mediated by plastic changes of cortical and cortico-muscular rhythms at the dominant frequency of human brain.

STRUCTURAL, FUNCTIONAL AND REGULATORY MUSCLE MOLECULES

SEARCHING FOR SPECIFIC SIGNALS TARGETING TRIADIN AND JUNCTOPHILIN TO THE JUNCTIONAL REGION OF THE SARCOPLASMIC RETICULUM.

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The sarcoplasmic reticulum (SR) is a specialized form of endoplasmic reticulum responsible for calcium homeostasis in muscle cells. It is organized in specialized domains that include the terminale cisternae, that represent the junctional regions where the SR contacts the plasma membrane forming the triads and the longitudinal tubules. Many proteins have been described to be localized at the triads and to be involved in calcium release from the SR. These include the ryanodine receptors (RyRs) and the dihydropiridine receptors (DHPRs), the calcium binding protein calsequestrin and the regulating proteins triadin, junctophilin and junctin. Although the protein composition and the organization of the triads have been well described, little is known about the molecular mechanisms responsible for protein sorting to these specific subdomains. In order to identify minimal regions responsible for protein targeting to the junctional SR, we created a series of GFP tagged cDNAs coding for triadic proteins. In particular we focused our attention on triadin and junctophilin. Human triadin is a 729 a.a protein characterized by a short N-terminal cytoplasmic domain, a single transmembrane region and a long luminal domain. Triadin possessed two sites of interaction with ryanodine receptor a calsequestrin binding site and probably takes part in the macromolecular complex of calcium release. Junctophilin is a 661 a.a. protein characterized by a transmembrane domain at its extreme C-terminal and the rest of the protein that spans the cytoplasm between the T-tubule and the sarcoplasmic reticulum. It possessed two groups of MORN motifs (Membrane Occupation and Recognition Nexus) with which the protein can interact with the plasma membrane lipids. Full length GFP-fusion proteins as well as progressive deletion mutants of triadin and junctophilin have been expressed in primary rat myocites and their localization has been followed by confocal microscopy during differentiation.

MATURATION OF THE SR-MITOCHONDRIAL CONNECTIVITY IN DEVELOPING SKELETAL MUSCLE.

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In adult skeletal muscle, mitochondria are primarily located in proximity of Z lines, next to calcium release units (CRUs), or triads. However, limited information is available with regard to mitochondrial disposition during skeletal muscle development. We have studied the mitochondrial apparatus in *flexor digitorum brevis* (FDB) fibers from young (1-4 week old) and adult (2-4 months) mice using electron microscopy (EM). The EM analysis indicates that mitochondrial localization drastically changes during postnatal development: at 15 days postnatal, mitochondria are mostly found in longitudinally oriented clusters under the sarcolemma and between myofibrils of the muscle fiber. This disposition progressively changes up to a point in which, at 2-4 months after birth, most mitochondria are precisely targeted next to triads, between the edge of the A band and the Z line. We have quantified the number of mitochondria (and

triads) at the different developmental stages and also estimated the percentage of mitochondria located adjacent to triads at the I band. The results of these studies indicate that: 1) the number of both mitochondria and triads increases in parallel during the first two months of development; and 2) the percentages of mitochondria located at the I band and in proximity to the triad is also time-dependent and increases with age. These data indicate that the development of EC coupling and mitochondrial localization is a highly coordinated process. In addition, under higher magnification, electron dense structures appear to tether, or bridge, individual mitochondria to parajunctional regions of the triad and, more precisely, to the terminal cisterna that is closer to the Z line. At this stage we have no indication of the molecular nature of these tethers and can only speculate as to the possible structural and/or functional role that these structures may have in connecting Ca^{2+} stores/release with mitochondria.

MOLECULAR AND IMMUNOHISTOCHEMICAL STUDY OF SARCOGLYCANS IN NORMAL HUMAN SMOOTH MUSCLE.

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Sarcoglycan complex is composed of the subunits α, β, γ, δ, ε and ζ. First described in skeletal muscle, the complex is also present in smooth and cardiac muscles, although its composition varies according to the muscle type. Previous investigations have demonstrated that in skeletal and cardiac muscle, the SGC is a heterotetrameric unit constituted by the α, β, γ, and δsarcoglycans. Other authors demonstrated that the expression of α-sarcoglycan is restricted to striated muscle cells, whereas ε-sarcoglycan, is also expressed in several other tissues. Successive reports suggest that two sarcoglycan subcomplexes exist: one containing α, β, γ, and δ sarcoglycan, characteristic of skeletal and cardiac muscle, and other consisting of ε, β, γ, δ sarcoglycan peculiar to vascular smooth muscle (1). In this study, we performed an immunofluorescence semiquantitative analysis and molecular study using confocal laser scanning microscopy and RT-PCR on surgical biopsies of human adult gastroenteric, urinary and respiratory smooth muscle in order to verify whether the ε sarcoglycan replaces α sarcoglycan in smooth muscle to form a unique sarcoglycans subcomplex. Our results showed that in all observed samples of smooth muscle also α-sarcoglycan is always detectable, although its staining pattern is slightly lower than ε-sarcoglycan. Therefore, one intriguing possibility is the existence of a pentameric, or, also considering ζ-sarcoglycan showed in the RT-PCR, hexameric model of SGC which could present a larger or lower expression of single sarcoglycan in conformity with muscle type, skeletal, cardiac, or smooth, or also in conformity with the origin of smooth muscle, gastrointestinal, urogenital, or respiratory tract.

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ROLE OF ACTIN CYTOSKELETON IN THE MODULATION OF STRETCH-ACTIVATED CHANNELS SENSITIVITY OF MYOBLASTIC C2C12 CELLS.

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Since it is thought that stretch activated channels (SACs) are structurally and functionally coupled with cytoskeleton, in the present study we explored the short term effects of stress fibers (SF) formation on the conductive properties of SACs in C2C12 myoblasts.

To this purpose, the cells were cultured in the presence or absence of 1 μM sphingosine 1-phosphate (S1P) for 30 min, and treated with the actin disrupting agents, or with antibodies directed against focal adhesion proteins. In response to S1P stimulation, myoblasts developed a robust cytoskeleton, characterized by randomly oriented cell spanning SF connected with a prominent peripheral actin web. S1P-dependent cytoskeletal reorganization was associated with a significant increase in the amplitude of transmembrane ion currents through SACs and in plasma membrane stiffness as evaluated by AFM, compared to those recorded in unstimulated cells. Disruption of SF markedly enhanced SACs current while reducing plasma membrane stiffness in unstimulated and S1P-stimulated myoblasts, suggesting that cytoskeletal disassembly, was as effective as SF formation, in causing plasma membrane deformation and SACs activation. Moreover, microinjection with anti-vinculin, anti-cortactin, anti-FAK antibodies, did not substantially modified formation of SF in response to S1P whereas caused a dramatic enhancement of SACs current and a sharp reduction in the membrane rigidity.
Together, these results clearly showed that SF reorganization was an important determinant in the regulation of plasma membrane elasticity and, in turn, of ionic conductance through SACs in myoblastic C2C12 cells.

DIFFERENTIAL DISTRIBUTION OF HOMER ISOFORMS IN SKELETAL AND CARDIAC MUSCLES. SLOW-TWITCH SKELETAL MUSCLE FIBER TYPE SPECIFICITY OF HOMER 2A/B.

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Homer represents a new and diversified family of proteins made up of several isoforms. The presence of Homer isoforms, referable to 1a, 1b/c, 2a/b and 3, was investigated in fast- (F)and slow- (S) twitch skeletal muscles as well as in cardiac muscle (H), from both rat and mouse. Distribution of Homer isoforms was strikingly different: identical for Homer 1b/c and Homer 3, irrespective of the muscle, graded for Homer 1a, H>>>S>F, specific for Homer 2a/b that was virtually present in slow-twitch skeletal muscle only, i.e., Homer 2a/b is marker of the slow-twitch phenotype. Transition in Homer isoforms compositon was also studied upon denervation of slow-twitch skeletal muscles. No change of Homer 1a and Homer 1b/c was observed up to 14 days after denervation; on the other hand, Homer 2a/b was found to be significantly decreased at 1 day and diminished by 90% at 14 days after denervation. Homer 2a/b was also negatively and linearly related to both muscle mass and type 1 myosin heavy chain. Thus, Homer 1 isoforms are not implied in adaptive changes affecting slow-twitch muscles following denervation, whereas Homer 2a/b accompanies muscle atrophy and its decrease parallels the slow-twitch phenotype disappearance. The role of Homers in skeletal muscle plasticity – disuse atrophy, denervation atrophy, regeneration, differentiation – is growing stronger.

REGULATORY MECHANISMS OF MYOGENESIS

ROLE OF CX43 EXPRESSION INDUCED BY SPHINGOSINE 1-PHOSPHATE IN MYOBLAST DIFFERENTIATION.

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Sphingosine 1-phosphate (S1P) is a bioactive lipid believed to be an effective regulator of skeletal muscle biology, acting as a prodifferentiating and physiological anti-mitogenic agent. At present, its downstream effectors are poorly known. In this study we provide experimental evidence for a novel mechanism by which S1P regulates skeletal muscle differentiation through the regulation of gap junctional protein

connexin (Cx)43. In fact, the treatment with S1P significantly enhanced Cx43 expression as well as gap junctional communication between myoblasts during the early phases of muscular differentiation. In contrast, the down-regulation of Cx43 by transfection with short interfering RNA blocked myogenesis elicited by S1P. By using different experimental approaches, we have demonstrated that calcium and p38 MAPK-dependent pathways were required for S1P-induced increase in Cx43 expression. Interestingly, enforced expression of mutated Cx43(Delta130-136) reduced gap junction communication and totally inhibited S1P-induced expression of the myogenic markers, myogenin, myosin heavy chain, caveolin-3, and myotube formation. Particularly, in S1P-stimulated myoblasts, endogenous or wild type Cx43 protein, but not the mutated form, coimmunoprecipitated and colocalized with F-actin and cortactin in a p38 MAPK-dependent manner. Our results, together with the known role of actin remodelling in cell differentiation, strongly support the important contribution of gap junctional functional communication, Cx43 expression and Cx43/cytoskeleton interaction in skeletal myogenesis induced by S1P.

$MnCl_2$ EFFECT ON MYOGENESIS: AN IN VITRO STUDY ON C2C12 CELLS AT VARIOUS DIFFERENTIATION STAGES.

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Manganese (Mn) is a trace element, essential for many metabolic functions and cell homeostasis; in excess, however, it is also a tissue-/cell-specific toxicant, whose adverse effects emerged, firstly, in central nervous system. Typical signs of Mn toxicity include, in fact, neurobehavioral disorders and extra-pyramidal motor dysfunctions (*manganism* syndrome). Even if widely investigated, the molecular mechanisms of Mn toxicity is still unknown; in particular, the exact relation between the Mn-induced deficit in muscle function and the neurotoxic effect of the metal remains to be clarified. A potential direct toxic effect of Mn on skeletal muscle has long been hypothesised, since Mn can accumulate in muscle tissue and interfere with calcium-regulated ionic currents in cell membranes. Previous results recently obtained by our group also suggested a direct cytotoxic effect of Mn on skeletal myocytes (Rovetta *et al.*, *Toxicol in Vitro*, 2006, in press).
In the present work C2C12 muscle cells at various differentiation stages (myoblasts and myocytes/myotubes) were treated for different time periods (1 to 7 days) with increasing $MnCl_2$ concentrations (0.1 μM to 1 mM). Cell viability, proliferation rate and fusion index were used as parameters to assess Mn toxicity; fluorescent markers specific for either apoptosis or necrosis processes were employed to integrate the study of Mn effect on cell cycle progression. The study was completed with morphological analyses (optical microscopy).
Taken together, our results demonstrated that, in C2C12 cells, $MnCl_2$ treatment directly affects all the considered biological parameters, being the observed effects dose- and time- dependent and also clearly related to the considered cellular differentiation stage (myoblasts *versus* myocytes/myotubes response). Even if preliminary, our findings seem then to support the hypothesis of a possible involvement of a direct myopathy in the pathogenesis of the Mn-induced motor system impairments.

ENFORCED EXPRESSION OF RAGE IN RHABDOMYOSARCOMA CELLS RESULTS IN REDUCED PROLIFERATION, MIGRATION, AND INVASIVENESS IN VITRO, ACTIVATION OF A MYOGENIC PROGRAM, AND REDUCED TUMOR GROWTH IN VIVO.

Francesca Riuzzi, Guglielmo Sorci and Rosario Donato

Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma, arising from muscle precursor cells. TE671 RMS cells, like other RMS cell lines, show inability to complete the differentiation program despite the expression of muscle-specific regulatory proteins. However, persistent activation of p38 MAPK in RMS cells results in growth arrest and myogenic differentiation (*Genes Dev*

14, 574-584), implying that defective activation of p38 MAPK might be one cause of inability of TE671 cells to escape from the cell cycle and activate the myogenic program. RAGE (receptor for advanced glycation end products), activated by its ligand, amphoterin (HMGB1), stimulates myogenesis via a Cdc42-Rac1-MKK6-p38 MAPK pathway (*Mol Cell Biol* 24 (2004) 4880-4894), and functional inactivation of RAGE in myoblasts results in reduced myogenesis, increased proliferation and tumor formation in vivo (*J Biol Chem* 281 (2006) 8242-8253). We show here that TE671 RMS cells, which do not express RAGE, can be induced to differentiate upon transfection with RAGE (TE671/RAGE cells) but not a RAGE mutant lacking the transducing domain (RAGEΔcyto) (TE671/RAGEΔcyto cells) via a Cdc42-Rac1-MKK6-p38 MAPK pathway, and that TE671/RAGE cell differentiation depends on RAGE ligation by amphoterin. TE671/RAGE cells also show reduced proliferation, migration and invasiveness and increased apoptosis, volume and adhesiveness in vitro, and generate smaller tumors with a lower incidence in vivo compared with wild-type TE671 cells. Two other RMS cell lines, CCA and RMZ-RC2, that do express RAGE, show an inverse relationship between the level of RAGE expression and invasiveness in vitro, and show reduced myogenic potential and enhanced invasiveness in vitro when transfected with RAGEΔcyto. These data support the proposal that RAGE ligation by amphoterin might play a physiological role in myogenesis and suggest that deregulation of RAGE expression in myoblasts might concur in rhabdomyosarcomagenesis.

S100B PROTEIN ACTIVATES QUIESCENT MYOBLASTS AND MUSCLE SATELLITE CELLS.

Guglielmo Sorci, Francesca Riuzzi and Rosario Donato

Treatment of high-density myoblast cell lines or primary myoblasts (satellite cells) in differentiation medium (DM) with pM amounts of S100B results in inhibition of differentiation, stimulation of proliferation and inhibition of apoptosis (*Mol Cell Biol* 23:4870-4881; *J Cell Physiol* 207:461-470). Thus, S100B might participate in embryonic myogenesis and muscle regeneration by increasing the myoblast/satellite cell number and by modulating myotube/myofiber hypertrophy. S100B stimulated myotube formation when given to low-density myoblasts in DM for 24 h followed by a 72-h cultivation in the absence of S100B, and administration of S100B for 24h to myoblasts/myotubes that had been cultivated for 72 h in DM resulted in myotube hypertrophy. These latter results suggest that S100B might activate non-fused, quiescent (G_0-arrested) myoblasts and stimulate their proliferation, thereby promoting myotube formation/hypertrophy. To support this possibility, we made myoblasts quiescent, switched them to DMEM for 16 h and analyzed them for cell cycle by FACS. S100B decreased the fraction of myoblasts in G_0/G_1 phase and increased the fraction of myoblasts in S phase. These events were accompanied by a rapid activation of ERK1/2, p38 MAPK and Akt, and in a typical time-course of changes in the levels of the muscle-specific transcription factors Myf5 and MyoD (*J Cell Biol* 142:1447-1459). However, a long exposure (i.e., 3-4 days) of low-density myoblasts to S100B resulted in inhibition of differentiation, similar to a brief (24 h) exposure to S100B of high-density myoblasts (with stimulation of ERK1/2 and inhibition of p38 MAPK). The finding that S100B can activate quiescent myoblasts and satellite cells suggests that the protein might contribute to regulate embryonic myogenesis and muscle regeneration by stimulating proliferation of low-density myoblasts/satellite cells and by reducing the myogenic potential of high-density myoblasts/satellite cells by its proliferative stimulus.
Analyses are in progress to dissect the molecular mechanism whereby S100B exerts such a dual role in myoblast differentiation. However, the finding that S100B can activate quiescent myoblasts and satellite cells suggests that the protein might contribute to regulate embryonic myogenesis and muscle regeneration, stimulating proliferation and migration of low-density myoblasts/satellite cells and reducing the myogenic potential of high-density myoblasts/satellite cells.

EFFECT OF SPHINGOSINE 1-PHOSPHATE ON PROLIFERATION AND SURVIVAL OF MURINE MESOANGIOBLASTS.

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Mesoangioblasts are mesodermal progenitors isolated from the dorsal aorta in mammals. They grow easily in vitro and, due to their self-renewal ability, are considered a new class of stem cells. Being capable of contributing to muscle regeneration in animal models, these cells are promising in the therapy of muscular dystrophy. The characterization of the agonists capable of regulating proliferation and survival of mesoangioblasts is fundamental to improving their employment in cell therapy.
Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid, product of sphingomyelin metabolism, physiologically present in plasma. It is able to regulate biological processes such as proliferation, cell migration and apoptosis in different cell types. Initially identified as a second messenger of growth factors, it is now clear that most of the effects elicited by S1P are due to its ligation to specific G-protein coupled receptors, named S1PR. Up to now five different S1PR, $S1P_{1-5}$ have been identified. These almost ubiquitously expressed receptors couple to different G-proteins, mediating the activation of distinct signalling pathways.
Here we provide evidence that mesoangioblasts express three S1PR on their surface. They are able to activate S1P-mediated signalling pathways and also to produce S1P, since they express sphingosine kinase, which catalyze the phosphorylation of sphingosine into S1P. Moreover, S1P (1 μM) potently stimulated mesoangioblast cell proliferation, evaluated by thymidine incorporation and cell counting experiments. By employing specific agonists and antagonists of S1PR, the receptor subtypes involved in the mitogenic action of the bioactive lipid have also been characterized. Interestingly, the sphingolipid was also found to protect mesoangioblasts from apoptosis induced by starvation, or treatment with staurosporine or C2-ceramide, assessed by activation of caspase-3, cell counting and determination of cytoplasmic histone-associated-DNA-fragments.

APOPTOSIS SUSCEPTIBILITY OF MYOGENIC CELL LINES EXPOSED TO SUPRA-PHYSIOLOGICAL CONCENTRATIONS OF GH AND IGF-1.

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During the past decade there has been an increase in the nontherapeutic use of anabolic steroids in the sport's world, especially in the late 1980s. Among prohibited substances, GH and IGF-1 are critical hormones for the regulation of postnatal growth and the maintenance of lean body mass in humans. The main goal of this study is to analyse the correlation between in *vitro* exposure of mouse and rat myoblasts to supra-physiological concentration of combined growth factors and the induction of DNA lesion and apoptosis by stress conditions.
As regards cellular response, we have tested myogenic cell lines, C2C12 and L6C5, after the exposure to supra-physiological concentration of growth hormone (GH or IGF1) in combination with apoptotic cell death inducers (hydrogen peroxide, ethanol). We show that these anabolic steroids could be involved into the damage-induced response. In fact, the addition of exogenous GH in medium determines different response to apoptosis-induced in both myogenic cell lines. Morphological determination of apoptosis with Hoechst show that exogenous GH combined with hydrogen peroxide increase cell death for either cell lines. When cells were treated with ethanol the effect was different. The cell death is significantly lower with treatment combined in relation to that observed with ethanol alone. Moreover, we have transfected

myogenic cell lines with pcDNA3-IGF1. In this model IGF1 could play an important role into the regulation of cell proliferation and apoptosis.
This study will suggest the biological effect of these hormones on the skeletal muscle and to identify a new class of markers specifically correlated to the cellular effect induced by hormone abuse.

MELATONIN AND UVB-INDUCED MUSCLE CELL DEATH.

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Several myopathies are characterized by muscle cell death, which causes muscular mass reduction and progressive atrophy (Tews 2005, Muscle Nerve. 32:443). Our intent is to investigate muscle apoptosis, and the possible protective role of melatonin, recently described in other apoptotic systems (Luchetti et al,2006,J Pineal Res.40:158).
In this study muscle death was experimentally induced in C2C12 murine myoblastic cell line by utilizing UVB-irradiation. This trigger generates oxidative stress in mammalian cells through an increased production of reactive oxygen species, determining irreversible and reversible cell changes (Somosy,2000,Micron.31:165).
Monolayers were grown as previously described (Burattini et al, 2004, Eur J Histochem.48:223). For apoptosis triggering, they were irradiated for 30 min and postincubated for 4h in 5%CO_2 at 37°C. After UVB irradiation the medium was supplemented with 1mM melatonin.
Cytotoxicity was determined with MTT assay and morphological apoptotic patterns were investigated with transmission and scanning electron microscopy (Sestili et al,2006,Free Radic Biol Med.40:837).
Cytotoxicity analysis of UVB-irradiated cells shows that viability considerably decreases. Differently, after melatonin postincubation, it increases, restoring a cell condition similar to control one. Morphological investigation, in irradiated cells, reveals apoptotic features and bleb formation, as well as an intriguing increase of substrate anchorage, possibly correlated to cytoskeleton role in UVB cell response. Melatonin seems to prevent these phenomena.
Further studies are in progress to highlight the modified expression of cytoskeletal muscle proteins after UVB treatment as well as after melatonin postincubation. The protective effect of the hormone will be also consistently investigated, in order to hypothesize a new potential therapeutic role.

MOLECULAR MECHANISMS OF MUSCLE ATROPHY.

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Skeletal muscle is the most abundant tissue in the human body accounting for almost 50% of the total body mass and it is a major site of metabolic activity. Since muscle is the largest protein reservoir, it serves as a source of amino acids to be utilized for energy production by various organs during catabolic periods. For instance, amino acids generated from muscle protein breakdown are utilized by the liver to produce glucose and to support acute phase protein synthesis during fasting. A number of catabolic disease states, including sepsis, burn injury, cancer, AIDS, diabetes, and renal failure, neurological disorders are characterized by muscle wasting, mainly reflecting increased breakdown of myofibrillar proteins and loss of organelle including mitochondria. The recently discovery of two muscle specific ubiquitin ligases, atrogin-1/Mafbx and Murf1 that are necessary for muscle loss, has begun to define the individual Ubiquitin-Proteasome components critical to the atrophy process. The expression of these Ub-ligases increases 8-40 fold in all types of atrophy studied and precedes the onset of muscle weight loss. We and others have recently defined that these ubiquitin ligases and protein breakdown in general are blocked by the growth-promoting IGF1/PI3K/AKT pathway. The FoxO family, a downstream target of

AKT, was identified as the main transcription factors regulating not only atrogin-1 expression but also an atrophy program which leads to a loss of muscle mass. FoxO family members have been shown to regulate various cellular functions including apoptotic cell death. Here we investigated the possible role of FoxO in inducing mitochondria damage and activation of autophagy pathways. Overexpression of constitutive active Foxo3 in adult muscle fibers causes a reduction in mitochondrial content, apparently due to their destruction in autophagic vacuoles. The fact that FoxO controls mitochondria and muscle atrophy prompted us to investigate whether expression of PGC1α, the master regulatory genes for mitochondria biogenesis, can block muscle loss. Coexpression of PGC1α reduced the capacity of Foxo3 to cause fiber atrophy and helps maintain mitochondrial content. Thus mitochondria damage/loss and energy balance become critical steps in muscle wasting.

AGING, MUSCLE DISEASES AND REGENERATIVE PATHWAYS

CLINICAL PHENOTYPE AND FUNCTIONAL CHARACTERIZATION OF CASQ2 MUTATIONS ASSOCIATED WITH CATECHOLAMINERGIC POLYMORPHIC VENTRICULAR TACHYCARDIA.

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Four distinct mutations in the human cardiac calsequestrin gene (*CASQ2*) have been linked to catecholaminergic polymorphic ventricular tachycardia (CPVT). The mechanisms leading to the clinical phenotype are still poorly understood because only 1 *CASQ2* mutation has been characterized in vitro.
We identified a homozygous 16-bp deletion at position 339 to 354 leading to a frame shift and a stop codon after 5aa (CASQ2 $^{G112+5X}$) in a child with stress-induced ventricular tachycardia and cardiac arrest. The same deletion was also identified in association with a novel point mutation (CASQ2^{L167H}) in a highly symptomatic CPVT child who is the first CPVT patient carrier of compound heterozygous *CASQ2* mutations. We characterized in vitro the properties of *CASQ2* mutants: CASQ2 $^{G112+5X}$ did not bind Ca^{2+}, whereas CASQ2 ^{L167H} had normal calcium-binding properties. When expressed in rat myocytes, both mutants decreased the sarcoplasmic reticulum Ca^{2+}-storing capacity and reduced the amplitude of *I*Ca-induced Ca2+ transients and of spontaneous Ca^{2+} sparks in permeabilized myocytes. Exposure of myocytes to isoproterenol caused the development of delayed afterdepolarizations in CASQ2$^{G112+5X}$.
CASQ2 ^{L167H} and CASQ2 $^{G112+5X}$ alter CASQ2 function in cardiac myocytes, which leads to reduction of active sarcoplasmic reticulum Ca^{2+} release and calcium content. In addition, CASQ2 $^{G112+5X}$ displays altered calcium binding properties and leads to delayed afterdepolarizations. We conclude that the 2 *CASQ2* mutations identified in CPVT create distinct abnormalities that lead to abnormal intracellular calcium regulation, thus facilitating the development of tachyarrhythmias.

ORAL AMINO ACID SUPPLEMENTATION COUNTERACTS SARCOPENIA IN THE SOLEUS MUSCLE OF ELDERLY RATS.

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The present study investigated the effects of oral amino acid supplementation on the adaptational changes induced by ageing in the soleus muscle of the rat. Male Wistar rats were divided in three groups: control

young (YO, 3 mo of age), control elderly (EL, 20 mo of age) and EL orally supplemented with amino acid mixture (Aam, EL-AA, 18 mo of age, BigOne® 1mg/gr/day in drinking water for 8 wk). Myosin Heavy Chain (MHC) composition was analyzed in all muscles. Total number (TFN) of type 1 and 2A fibers and fiber cross sectional area (CSA) was also measured in immunostained sections. The sarcomere (Vsar/Vtot) and mitochondrial density (MiN/100μ^2) of single muscle fibers were studied by electron microscopy. The expression of total and phosphorylated serine/threonine protein kinase mTOR and p70 S6K1, potent regulators of mRNA translation initiation, were also determined in all groups.
Ageing was associated with atrophy of fast and slow fibers, a significant decrease of Vsar/Vtot and MiN/100μ^2, no changes in TFN and an overall shift towards the expression of a slower MHC phenotype.
Aam supplementation antagonized the effects of ageing as an increase of fibers CSA, Vtot/Vsar, MiN/100$\mu^2$and a slight slow-to-fast shift of MHCs were observed in EL-AA. Furthermore the level of activation of mTOR and p70 S6K1 appeared to be increased by Aam supplemetation.
Collectively these results suggest that ageing-induced muscle adaptations can be partly restored by amino acid supplements. An mTOR signal pathway may mediate the effects on fiber trophism.

PROTEOMIC ANALYSIS OF SOLEUS MUSCLE IN STZ-DIABETIC MICE: EFFECTS OF ORAL AMINO ACID SUPPLEMENTATION.

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Diabetes mellitus is a metabolic disorder leading to unbalance between protein synthesis and breakdown in the skeletal muscle. Amino acids (Aa), on the contrary, stimulate protein synthesis. We investigated the protein profile of the soleus muscle in streptozotocin (STZ)-induced diabetic mice with or without oral Aa supplementation in order to understand which skeletal muscle proteins were changed by both conditions. Four groups of mice were considered: control (C), amino acid supplementation (AA, 0.1 g/Kg/day for 15d), diabetes (D, STZ 65 mg/Kg), diabetes+Aa (DA).
Proteomic maps were generated by using 2-D electrophoresis and more than 500 protein spots on each gel were detected by silver staining. The analysis showed a general protein up-regulation in AA mice and a protein down-regulation in D.
Interestingly, we found a different expression in antioxidant defense system superoxide dismutase (SOD1) and heat shock proteins (HSPs: HspB1, Similar to Hsp 20 kDa and HspB7). Aa supplementation was associated with a statistical increase of SOD1 and no changes in HSPs expression. Diabetes induced decrease of SOD1 and increased cellular stress as demonstrated by overall up-regulated HSPs. Aa administration to D counteracted the effects of diabetes by producing an up-regulation of SOD1 and down-regulation of HSPs. Our data suggest a role of Aa supplementation in controlling the antioxidant defence system and reducing the oxidative stress in diabetic skeletal muscle.

PHYSIOLOGICAL AGEING ALTERS THE ABILITY OF HUMAN SATELLITE CELLS IN DAMAGE REPARING.

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Skeletal muscle fibres undergo a progressive loss of mass and strength during the ageing process defined sarcopenia. One of the factors that could play a key role in triggering sarcopenia is the accumulation of ROS in aged skeletal muscle. Our previous works showed an increase in oxidative stress, due to a decreased activity of some scavenger enzymes, both in muscular biopsies and in satellite cells derived

from aged donors. Oxidative damage could affects the satellite cells as well, also altering the functionality of Ca^{2+}-regulatory homeostasis proteins. Indeed, both in undifferentiated (myoblast) and differentiated (myotube) phenotype the basal levels of $[Ca^{2+}]_i$ is increased as already reported in literature for the muscle tissue.
In the present work we investigate the effects of sarcopenia and oxidative stress on myoblasts and myotubes derived from human donors, characterizing them from both the morphological and functional point of view. We found that myoblasts from aged individuals show an altered ability to fuse with each other and to get well differentiated myotubes. Moreover we check the appearance of functional receptors in our in vitro differentiated myotubes, so living myotubes was stimulated with Caffeine and KCl. We found functional DHP and Rya receptors already in 6 days in vitro differentiated myotubes. However we found an external Ca^{2+}-dependent e-c coupling in myotubes derived from aged individuals, that could be due to a probable disorganization of DHPR-RyR. These data are also sustained by the immunofluorescence experiments where we noticed a partial DHPR-RyR uncoupling. Finally we show an increase in lipid oxidative damage in myotubes from aged donors, that could be on of the causes, at least in part, of the altered differentiative program.
These data suggest that satellite cells from aged individuals present an altered differentiative program from both the morphological and functional point of view; this effect could be one of the causes for their reduced ability of damage repairing with the ageing.

A NEW ANIMAL MODEL OF SKELETAL MUSCLE AND NERVOUS TISSUE INTERPLAY: THE MLC/SODG93A MICE.

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Amyotrophic Lateral Sclerosis is a late onset neurodegenerative disease involving upper and lower motorneurons. Transgenic mice ubiquitously overexpressing human SOD1 mutants develop the pathological features of ALS, that are the degeneration and loss of motor neurons with astrocytic gliosis and intra neuronal inclusions in degenerating neurons and glia. Until recently, the strong relation between muscle and nerve has not been seriously considered as a potential target to develop a new therapy for ALS disease. Survival factors coming from the muscle to the nervous tissue could influence neuron survival, axonal growth and maintenance of synaptic connection. We recently described how the muscle specific overexpression of IGF1, can delay the onset and the progression of the disease in the SOD^{G93A} mice, the animal model of ALS, preserving muscle tissue from atrophy and inducing motor neuron survival. In light of these evidence skeletal muscle appears as a untested component in the study of ALS disease, and for this reason we generated a new transgenic animal, the MLC/SOD^{G93A} mice, overexpressing SOD1 mutant gene under the control of the muscle specific Myosin Light Chain promoter. Although the animals do not show any signs of ALS disease, we observed an evident atrophy of both slow and fast fibers associated with a reduced Tetanic force, and with an enhanced tendency to muscle fatigue; further, we observed an evident shift in muscle fibers composition from slow to fast phenotype, resembling what is usually observed during the denervation process. Since ALS is characterized by a massive inflammation in the spinal cord we tested the levels of cytokines in both transgenic and control mice and we observed a significant increase of MCSF, TGFb and TNFa levels in the transgenic animals. In the light of these results the MLC/SOD^{G93A} mice appears as a new model of muscle-nerve interplay, able to demonstrate how skeletal muscle atrophy can impair the physiological status of nervous tissue.

OXIDATIVE STRESS INFLUENCES Ca^{2+} TRANSPORT SYSTEM IN SKELETAL MUSCLE OF TRANSGENIC MICE.

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Reactive Oxygen Species (ROS) play a primary role in the ageing process, especially in those tissues in which the generation of free radicals is more pronounced such as nervous system and skeletal muscle where they are able to act on Ca-channel capacity (Eu et al 2000 Cell 102: 499-509) modifing Ca^{2+} homeostasis and, as consequence of this many aspects of cellular function. Also for this reason, the unbalance between ROS generation and rimotion is also strongly associated with some physiological (sarcopenia) or pathological statii such as ALS (Fulle et al 2004 Exp. Gerontol. 39:17-24; Mahoney et al 2006 Muscle Nerve 33:809-816). The aim of this experimental project is to validate the use of two transgenic models in which the antioxidant system is genetically altered by the modification of SOD1, a pivotal detossificant enzyme, to study, in a cell-free system (Fulle et al 2003 Neuromusc. Disord. 13: 479–84), some aspects of E-C coupling.
Models:

- $SOD1^{G93A}$ mouse which has a general over expression of a human familial mutation in CuZn-SOD and develops a severe motor neuron disease similar to ALS.
- MLC/SOD^{G93A} that expresses SOD1 mutation selectively in skeletal muscle, showing muscle atrophy and alteration in muscle strength.

Our results show that in $SOD1^{G93A}$ muscles:
i) exists a statistically significant presence of oxidative damage for lipides and proteic substrates (even if the enzimatic detossificant activity is radically increased)
ii) the number and the activity of DHPR channels and the Sarcoplasmic Ca^{2+} pump activity decrease.
In contrast, in MLC/SOD^{G93A} muscles we revealed:
i) an increase activity of detossificant system (mainly SOD and Catalase activity) without significant damage of biological molecules;
ii) an increase in the capacity of DHPR channels to shift in open status without any modification of protein expression.
In conclusion it is possible to hypothesize that the functional capacity of DHPR (Voltage-dependent L-type Ca-channel) organized in the tetrads in the T-tube is the functional target of oxidative stress in skeletal muscle.

REACTIVE OXYGEN SPECIES DETERMINE SKELETAL MYOBLAST ADAPTATION TO OXIDATIVE STRESS THROUGH NFKB ACTIVATION AND EXPRESSION OF PROTEIN INVOLVED IN CELL SURVIVAL AND DNA REPAIR.

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The aim of our study was to utilize a cellular model represented by L6C5 rat skeletal myoblasts to investigate the molecular mechanisms which determine the positive adaptation of the skeletal muscle to Reactive Oxygen Species (ROS).. The first aim of this study was to verify if the peroxide radical had a direct role in the activation of the adaptive response to oxidative stress of L6C5 myoblasts and to determine the time course for the activation of the cellular systems involved in this process. The supplementation with exogenous ascorbic acid (AsAc, 100 μM) during the adaptive treatment determined the complete disappearance of the anti-apoptotic response if AsAc was added from the beginning of adaptation, but no effects when it was added only during the final stage of the conditioning (last 8 hr), indicating that the up-regulation of adaptive genes is an early event during adaptation and specifically

requires low ROS concentrations. We found that ROS preconditioning increases the expression of both the Bcl2 and the αB-crystallin genes: αB-crystallin increased up to 6 hr from the end of adaptive treatment, returning to basal levels after 12 hr. Instead, the product of the Ref-1 gene, which plays a central role in DNA repair caused by ROS damage, increased only after 12 hr from preconditioning, in association with the induction of chromosomal breakages. Since it is known that L6C5 cells develop apoptosis resistance through modulation of the redox-sensitive transcription factor NFkB, we analyzed the modification of this factor activity under the adaptive treatment. Our results demonstrate a strong enhancement (8-fold increase over the control) of the NFkB activity after 24 hr of preconditioning with low ROS concentration, suggesting a central role for this transcription factor in the activation of the specific adaptation of skeletal muscle cells to oxidative stress.

EFFECT OF AGE IN Sgca-NULL HINDLIMB MUSCLES.

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Defects in the α-sarcoglycan gene (Sgca) cause the severe human type 2D limb girdle muscular dystrophy (LGMD-2D). Sgca-null mice are a valuable animal model to investigate the pathophysiological mechanism as they develop progressive muscular dystrophy similar to human disorder. Aging determines profound and progressive modifications of normal muscle and the present work was aimed at investigating the muscle aging in Sgca-null mice.
We compared the contractile properties of hind-limb muscles from adult (6 months) and old (15-18 months) control and Sgca-null animals. Hind limb muscles showed clear progressive pathological signs, with hypertrophic central nucleated fibers together with small regenerating ones. In control mice twitch contraction time increased in soleus and decreased in EDL with age, while they did not change in Sgca-null mice. The maximum rate of rise of tetanus decreased with age both in soleus and EDL control and dystrophic muscles. During aging, control EDL and soleus muscle showed a moderate decrease of specific twitch and tetanic tension, which were more pronounced in the Sgca-null muscles. In vivo measurements of force developed by gastrocnemius, stimulated via nerve, confirmed that Sgca-null muscles display a lower specific tension than controls, making evident a non-functional hypertrophy of dystrophic muscles. Tension decline during fatigue protocols did not differ between control and Sgca-null muscles, regardless the age, even though dystrophic EDL of Sgca-null mice was more resistant to fatigue than control. Grip test showed in control mice an age-related decrease in force development during voluntary contractions in vivo, whereas no significant age-related change was evident in Sgca-null mice.

FUNCTIONAL CHARACTERIZATION OF *Sgca*-NULL DYSTROPHIC DIAPHRAGM MUSCLE DURING AGEING.

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α-Sarcoglycan is a transmembrane glycoprotein of the dystrophin complex located at skeletal and cardiac muscle sarcolemma. Defects in the α-sarcoglycan gene (Sgca) cause the severe human type 2D limb girdle muscular dystrophy (LGMD-2D). Sgca-null mice develop progressive muscular dystrophy similar to human disorder, thus being a valuable animal model to investigate the physiopathology of the disorder. Elderly determines profound and progressive modification to normal muscle; present work was aimed at

investigate the functional changes in dystrophic muscle. We compared the contractile properties of diaphragm from young (3 months), adult (6 months) and old (15-18 months) control and Sgca-null animals. Control mice have a robust growth in the first 8-9 months that then progressively slows down. In contrast, Sgca-null mice, heavier in the first months compared to controls, progressively loose weight after the 9th month. Sgca-null diaphragm presents clear pathological signs that progressively worsen with age. Oxidative capacity of control diaphragm progressively increases from young to old, while it is already high in the young Sgca-null, which, however, presents large anomalous mitochondria distribution. Twitch and tetanic characteristics are not affected by aging and dystrophy. Conditions that did not fatigue control diaphragm, regardless the age, caused a significant drop of tension in the Sgca-null muscle. In addition, dystrophic diaphragm showed an unusual higher capacity of recovery from fatigue. The work demonstrates that respiratory muscles of Sgca-null mice undergo a progressive degeneration that mainly compromises resistance to fatigue.

TRANSCRIPTIONAL PROFILE IN HUMAN VASTUS LATERALIS OF PATIENTS AFFECTED BY CHRONIC FATIGUE SYNDROME.

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Chronic fatigue syndrome (CFS) is characterized by severe disabling fatigue and specific symptoms. For this pathology there is not yet a defined etiology and the only demographic risk factor is gender. It has been already demonstrated that in vastus lateralis muscle of CFS patients the oxidative damage to DNA and lipids increases. We studied the trascriptoma of vastus lateralis in CFS female and male patients out to define the possible disregulated genes and/or pathways. The aim was to document the origin of oxidative stress and to find some specific markers or clues that could help to understand the syndrome or suggest further focused investigation.
We utilised high-density oligonucleotide arrays for analysis of gene expression profile. We applied the Lowess (Locfit) normalization to expression data. After, log2 transformation was performed for each expression level. Identification of differentially expressed genes was performed with Significance Analysis of Microarray (SAM).
We selected the genes differentially expressed both on female and male patients and we accurately analysed them. In our opinion down-regulation of mithocondrial genes as SOD2, ferredoxin, NADPH dehydrogenase quinone 1 are responsible for reactive oxygen species accumulation and DNA damage. The up-regulation of □-polymerase could justify why the CFS symptoms are cyclic. In fact, the oxidative damage could be individually managed. The general down-regulation of MAPK pathway is probably linked to silencing the apoptotic/proteolitic tissue turnover. We documented also the down-regulation of the focal adhesion pathway and cytoskeletal reorganization.
In conclusion we think that exists a close relationship between oxidative damage and main symptoms of CFS. The main site of oxidative damage could be the mitochondrion that is unable to repair its DNA oxidation. We think that the oxidative damage management is different between the female and male gender and it depends also on individual features.

MAGIC F1, A DIMERIZED C-MET BINDING DOMAIN, INDUCES MUSCLE HYPERTROPHY BY PROTECTING FROM APOPTOSIS MYOGENIC PROGENITORS.

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Hepatocyte growth factor (HGF), also referred to as scatter factor (SF), is an important paracrine mediator of epithelial-mesenchymal cell interactions (*Birchmeier C & Gherardi E*). It is secreted by mesenchymal cells and affects cell proliferation, motility and morphology. These diverse biological activities are a result of HGF/SF binding to and activating its high affinity tyrosine kinase receptor called c-Met. HGF/SF was the first growth factor shown to be able to stimulate activation and early division of adult satellite cells in culture and in muscle tissue. MAGIC F1 (cMet activating genetically improved chimeric factor 1) is a human recombinant protein HGF-related, containing the leader sequence for protein secretion, an hairpin loop sequence and a combination of double repeated NK1 and NK2 functional kringle domains, where is located the high-affinity binding site for c-Met. It is able to activate c-Met receptor, stimulate AKT pathway and induce myocyte hypertrophy both in vitro and in vivo. Moreover, the AKT phosphorylation preserves myocytes from apoptotic events during muscle regeneration as revealed in a transgenic animal model expressing MAGIC F1 under the dependence of a skeletal muscle-specific promoter. These results showed clearly a starting point for clinical implication of MAGIC F1 recombinant protein, since inducing muscle hypertrophy could be a strategy to oppose the degenerative process of muscular pathologies.

Birchmeier C & Gherardi E. (1998) Trends Cell Biol. Oct;8(10): 404-10. Review

NEUROMUSCULAR PROPERTIES OF *DROSOPHILA* SPHINGOSINE-1-PHOSPHATE LYASE GENE (*SPLY*) MUTANTS.

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Sphingolipids play an important role in regulating cell death, survival, differentiation, migration (as well as a role in synaptic vescicle recycling). Sphingosine-1-phosphate regulates E-C coupling in mammalian skeletal muscle fibres and reduces muscle fatigue. Homologs of key enzymes belonging to the sphingolipids metabolic pathway have been found in *Drosophila melanogaster*, making this animal a useful model for studying the functional role of these enzymes and their products.. Sphingosine-1-phosphate lyase catalyzes the conversion of sphingosine-1-phosphate to ethanolamine phosphate and a fatty aldehyde. In previous studies we have shown that sphingosine-1-phosphate lyase gene (*Sply*) mutant adult flies have a reduction in flight performance. The question we address in the present sudy is whether there are changes in the function of the nerve-muscle system that could contribute to this flight performance defect. Using sply IIIrd instar larva body wall preparations, it was observed that locomotor behaviour was reduced with respect to controls. Specific tension measurements on muscle 6-7 showed a reduction of force output when muscle was indirectly stimulated through the nervous system with a tetanus. No changes were observed when muscle was directly stimulated either electrically or pharmacologically. Electrophysiological recordings showed that sply NMJ spontaneous and evoked neurotransmitter release was altered with respect to control flies. We conclude that the defects in sphingolipid synthesis in sply mutants are associated to functional changes in the the nerve-muscle system.

PRESENCE OF NEUROTROPHIC FACTORS IN SKELETAL MUSCLE CORRELATES WITH SURVIVAL OF SPINAL CORD MOTOR NEURONS.

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As developmental biologists, we employ heterozygous, *Myf5-/-*, *MyoD-/-* and *Myf5-/-:MyoD-/-* (N.B., myogenic regulatory factors) embryos, that contain myogenic precursos cells (MPCs) at different levels of commitment or completely lack all skeletal muscles, to test *in vivo* neurotrophic requirements of motor and proprioceptive neurons that innervate MPCs and muscle spindles, respectively. While all of these neurons are completely eliminated in amyogenic embryos, all of the neurons are intact in heterozygous, *Myf5-/-* and *MyoD-/-* embryos. For instance, our results indicate that MPCs for the limb musculature (affected in *MyoD-/-* mutants) express NT-3 and GDNF but not BDNF and NT-4/5, indicating that BDNF and NT-4/5 are not necessary for survival of lateral motor column neurons (i.e., those innervating the limb or MyoD-dependent MPCs). To determine which (neurotrophic factor) NF is important for survival of a certain group of motor neurons, we assessed the expression pattern of muscle-secreted NFs (e.g., BDNF, NT-3, NT-4/5 and GDNF) and their receptors in mutant and control neural tissues and MPCs. Furthermore, the expression pattern experiments are followed by the *in utero* treatment with a single NF or combinations of NFs. For instance, our results indicate that BDNF and NT-3 are important for survival of MMC neurons. To test this hypothesis, BDNF and NT-3 are injected *in utero* to attempt the rescue of MMC neurons in *Myf5-/-:MyoD-/-* embryos. Following the *in utero* treatments, embryos are processed for immunohistochemistry against Islet-1/2 and HB9 (specific spinal motor neuron markers for assessing the number of motor neurons), PCNA (proliferating cell nuclear antigen for assessing the proliferating index) and TUNEL (apoptosis detection system for assessing the apoptotic index). The cell counts, the number of proliferating and apoptotic cells are measured to determine the extent of the neuronal rescue. Since our data indicate that different subclasses of motor neurons have different neurotrophic requirements for their survival, we are starting to understand the role of skeletal muscle in providing underlying mechanisms for survival and maintenance of muscle innervating neurons *in vivo*. Supported by Canadian Institutes for Health Research (CIHR) to B.K.

MUSCLE DISEASES AND REGENERATIVE PATHWAYS

TNF-α NEGATIVELY AFFECTS SKELETAL MUSCLE REGENERATION AND PERFORMANCE THROUGH A PW1-, HSP70-DEPENDENT CASPASE ACTIVATION.

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Impairment of skeletal muscle regeneration is associated with muscular and non-muscular diseases. TNF blocks myogenesis *in vitro*[2], inhibits muscle regeneration *in vivo* and induces muscle waisting[1]. To study the mechanism underlying TNF effects on skeletal muscle we treated regenerating *Tibialis anterior* with TNF. TNF delays and reduces muscle regeneration, by increasing caspase activity in interstitial cells expressing stem cell markers (i.e. Sca-1, CD34, CD45 and PW1). Inhibition of PW1-dependent caspase activation (by either overexpressing a PW1dominant negative form, ΔPW1, or by injecting the caspase inhibitor Z-VAD) rescues muscle regeneration in the presence of TNF. Treatment of regenerating muscle with the myogenic factor AVP[3] counteracts TNF negative effects on muscle regeneration. To evaluate if the outputs of muscle regeneration correlate with muscle performance functional analysis is performed. Both tetanic and specific muscle force correlate with the extent of regeneration: TNF diminishes muscle force and resistance to fatigue, while AVP increases muscle force, counteracting TNF negative effects on muscle performance. We show that Hsp70 is involved in the different response of regenerating muscle to

AVP and TNF, and that Hsp70 overexpression is sufficient to block TNF negative effects on muscle regeneration. We demonstrate that TNF negatively affects skeletal muscle regeneration and performance and that TNF effects can be rescued by pharmacological (Z-VAD), gene delivery (ΔPW1 or Hsp70) or hormonal (AVP) treatment.
1) Coletti D et al. Genesis. 2005;43(3):119-127. 2) Coletti D et al. EMBO J. 2002;21(4):631-42. 3) Scicchitano BM et al. Mol. Biol. Cell 2005;16:3632-41.

ROLE OF PKC THETA IN CARDIAC HOMEOSTASIS AND REMODELLING.

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Cardiac remodelling (CR) qualifies changes that result in the rearrangement of normally existing structures in response to variety of pathologic stimuli, including myocardial infarction, contractile abnormalities, increased workloads and pressure overload. CR is associated with increased cardiac mass, the accumulation of interstitial collagen and decreased cardiac contractility.
Remodelling is frequently preceded by pathological enlargement of the heart due to hypertrophy of cardiac myocyte. This hypertrophic growth is characterized by an increase in cell surface area of myocytes, enhanced sarcomeric organization and re-expression of specific "foetal genes" (β-MyHC, ANF e α-SkA).
The hypertrophy of cardiomyocytes has been associated with alterations in intracellular signal transduction pathways, including MAPKs, Calcineurin and PKCs.
The protein kinase C constitutes a family of serine/threonine kinases which plays distinguished and specific role in regulating heart homeostasis and hypertrophic growth. To investigate the role of PKCθ (calcium-independent but phospholipid-dependent) in cardiac hypertrophy and re-modelling we employed PKCθ–KO mice. Echocardiographic and hemodynamic analysis indicates that mice lacking PKCθ develop reduced ventricular performance (ipocontractility and systolic dysfunction).
Histological analysis revealed that KO mice display increased fibrosis and elevated expression of collagen, associated with a significant increase in size of cardiomyocytes. PKCθ ablation: 1) does not alter the levels of expression of the other isoforms; 2) interferes whit the levels of expression and/or activation of Akt, GSK3β and JNK1, and whit the intracellular localization of HDAC5, all factors involved in the regulation of cardiac hypertrophy and cell death; 3) is accompanied by re-expression of "cardiac foetal genes" (β-MHC, ANF and α-SkA). Moreover PKCθ ablation reduces the phenylephrine-induced hypertrophy in cultured cardiomyocytes.
Keywords: cardiac hypertrophy, PKC, fibrosis.

ROLE OF PKC THETA IN SKELETAL MUSCLE HOMEOSTASIS, AND REGENERATION .

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PKCθ, a member of "novel" calcium-independent PKCs, is the PKC isoform predominantly expressed in skeletal muscle: it is localized at the neuromuscular junctions, its expression is developmentally regulated and it is under a degree of neural control. We previously shown that the expression of a "kinase dead" dominant negative mutant form of PKCθ cDNA, PKCθ K/R, specifically in muscle of transgenic mice induces insulin-resistance associated to obesity, due to the impairment of insulin signal transduction (Serra et al. *J. Cell. Physiol.* 2003). In addition, using cultured myogenic cells, we recently demonstrated that PKCθ co-operates with calcineurin in the activation of slow muscle genes (D'Andrea et al. *J. Cell.*

Physiol. 2006). Using in addition a mouse strain in which the PKCθ gene has been inactivated by gene knock out, we now show that ablation of PKCθ activity/expression leads to a significant reduction of muscle mass, due to reduction of muscle fibre size. Moreover, when PKCθ null mice were crossed with the MLC/mIGF-1 (a transgenic model of muscle hypertrophy) limb muscle mass and cross sectional area of muscle fibres were clearly reduced when compared with mIGF1 littermates. In addition, we observed that muscle regeneration is delayed in PKCθ mutant mice, suggesting that activation/growing of satellite cells is impaired. Indeed, PKCθ mutants muscle primary cultures differentiate into thin, oligonucleated myotubes, compared to wt satellite cells. However, when PKCθ mutant satellite cells were cultured in differentiation medium conditioned from wt satellite cells, myotube size and myonuclei number were restored.

MOLECULAR MECHANISMS REGULATING THE MAINTAINING OF SKELETAL MUSCLE HOMEOSTASIS

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Following mechanical stress, protracted exercise or pathological injury, healthy skeletal muscle undergoes continuing tissue remodelling. Muscle homeostasis is played on the critical balance between necrotic/apoptotic phenomena, leading to an atrophic condition, and the regenerative processes, promoting muscle recovery. Skeletal muscle repair is a highly synchronized process involving the activation of many cellular responses which restore the morphological/functional characteristics of healthy skeletal muscle. Furthermore cachexia is characterized by a dramatic loss of muscle mass associated with a compromised muscle regenerative ability.
To study homeostatic processes we took advantage of Arg-Vasopressin (AVP), a potent myogenic differentiation factor. Here we demonstrate that the over-expression of V1a AVP receptor (V1aR) in muscle results in enhanced regeneration following experimentally induced muscle injury. V1aR over-expressing muscle exhibits: accelerated differentiation, early activation of regeneration markers and satellite cells, increased cell population expressing hematopoietic stem cell markers and its conversion to the myogenic lineage.
Moreover, in the animal model of tumor (C26)-induced cachexia, the V1aR over-expression strongly reduces the negative effects of cachexia on muscle repair showing a regenerative ability comparable to healthy muscle.
Furthermore our results suggest that calcineurin plays a key role in V1aR-dependent muscle regeneration by a mechanism involving the NFAT/IL-4 signaling pathway. In fact IL-4 is known to promote myoblast fusion and to be involved in the recruitment of mesenchimal stem cells.
This study highlights a novel *in vivo* role for the AVP-dependent pathway which may represent a potential gene therapy approach for many diseases affecting muscle homeostasis.

LOCAL EXPRESSION OF IGF-1 MODULATES THE RESOLUTION OF INFLAMMATORY RESPONSE AND ACCELERATES MUSCLE REGENERATION FOLLOWING INJURY

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Muscle regeneration following injury is characterized by myonecrosis with local inflammatory response, activation of satellite cells, and repair of injured muscle fibers. Persistence of inflammation often renders

the damaged muscle incapable of sustaining efficient muscle regeneration. Here we show that local expression of a muscle-restricted Insulin-like Growth Factor-1 (mIGF-1) transgene accelerates the regenerative process of injured skeletal muscle, modulating the inflammatory response and accelerating the remodeling of connective tissue.
At the molecular level, mIGF-1 expression significantly down regulated proinflammatory cytokines, such as tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta, and modulated the expression of CC chemokines involved in the recruitment of monocytes/macrophages. The rapid restoration of injured muscle, by mIGF-1 expression, was also associated with a modulation of key players of connective tissue remodeling such as smooth muscle actin (SMA), collagen, and interferon (INF)-gamma. By modulating the inflammatory response and limiting fibrosis, supplemental mIGF-1 creates a qualitatively different environment for sustaining more efficient muscle regeneration and repair. This represents an important advance in our understanding for the treatment of trauma in sports medicine and muscle disorders.
Keywords: mIGF-1, muscle regeneration, inflammation, cyto/chemokines.

STRONGLY MYOGENIC MACROPHAGE-SECRETED FACTORS SELECTIVELY STIMULATE A SUB-POPULATION OF PUTATIVE STEM CELLS AND ALLOW IN VIVO MUSCLE RECONSTRUCTION AFTER SUBTOTAL ABLATION.

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In the presence of macrophagic factors, small, round and poorly adhesive cells derived from adult rat muscle could be selected and expanded efficiently. These desmin- and MyoD-positive cells were highly myogenic, becoming gradually spindle-shaped and aligned before forming myotubes. They have properties already described in murine muscle stem cells and likely represent a new subpopulation of stem-like rat cells stimulated by macrophages, important to enhance muscle regeneration. Consistently, in rat the supply of macrophagic factors in vivo increased ten folds the amount of newly formed muscle after extensive muscle ablation. The same factors enhanced the in vitro proliferation of slow-growing myogenic cells. They also induced neonatal *pseudo-organotypic* cell cultures and adult myogenic cell preparations to produce large amounts of contracting myotubes and few foci of differentiation, respectively. Thus, the macrophage-sensitive stem-like cells appear to be more abundant in embryonic muscles. Macrophagic factors decreased the size of their stem-like target cells, a possible explanation being the induction of asymmetric divisions as previously proposed in activated satellite cells. We discuss the important hypothesis that macrophages can activate and preserve a subpopulation of myogenic stem cells during muscle regeneration.

MYOSIN MOTOR DINSFUNCTION IN DYSTROPHIC MOUSE MUSCLES:A STUDY ON PURE ISOFORMS.

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Duchenne muscular dystrophy (DMD) is a genetic diseases characterized by skeletal muscle wasting and atrophy (Allen et al., 2005). Recently it has been suggested that the impairment of skeletal muscle performance in DMD not solely depend on a loss of contractile muscle mass. Cross-bridge interaction was found to be impaired in strips of diaphragm muscles from the mdx mice (Coirault et al., 2000) and myosin extracted from bulk mdx diaphragm muscle was found to move actin filaments, in "in vitro motility assay"(IVMA), at lower velocity than control (Coirault et al., 2002) suggesting that function of the myosin molecule itself could be altered in dystrophic mouse muscles . However, the latter

experiments compared the function of a mixture of myosin isoforms. Despite non significant changes in the slow-to-fast MHC ratio, as compared with control, mdx mice diaphragm exhibited a shift from 2X to 2A and a reduction in 2B-MHC content so that the lower velocity observed could not be unequivocally related to an alteration of myosin. In the present study the "IVMA" was used to analyze the functional properties of pure myosin isoforms from mdx muscles to clarify whether the dystrophic process could affected myosin motor function. Soleus and gastrocnemius muscles were isolated from 6 months mdx male mice. Pure type 2B and 1 myosin isoforms were extracted from single fibres characterized for their MHC composition (Canepari et al 1999). Preliminary results show a decrease of velocity on type 2B (3.48(0.13 vs 4.02(0.19) while no differences were observed on type 1 myosin (0.84(0.04 vs 0.89(0.04). This suggested that the fast 2B myosin is selectively altered in dystrophic muscle, consistently with the idea of preferential involvement of fast isoforms in dystrophic process.

WETWARE DEVICES FOR CALCIUM AND BIOELECTRONICS STUDIES OF SINGLE CELLS WITH NON-INVASIVE STIMULATION AND REGISTRATION: CULTIVATION OF MUSCLE FIBERS AND MYOTUBES ON SEMICONDUCTOR CAPACITOR AND TRANSISTOR MICROCHIPS.

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The aim of this study was to develop an artificial silicon-muscle junction which allows the control of *in vitro* long term contraction patterns of individual cells to muscular tissue engineering, new experimental approaches, and muscular regeneration models.
Two distinct biological models were employed: 1) Satellite mouse cells, C2C12 lines and dissociated fibers were cultured on different silicon chip coated with autoassembling nanolayers and induced to differentiate to myotubes developing a muscle-silicon junction on capacitor spots integrated in the chip wire. This was used to elicit EC coupling on single myotubes monitored with calcium fluorescence dyes. 2) Fully differentiated mouse muscle fibers were cultured on chip devices for the same purposes and for non-invasive registration through Transducive-Extracellular-Potential (TEP) induced in the cleft junction between surface chip and fiber membrane as results of evoked action potentials.

Single cells can be stimulated and monitored in a precise and targeted way and cells of the same Petri dish exposed for long term inside incubator under different stimulation patterns can be compared. This model is used to study muscular plasticity at the artificial junction level.
The support of the Telethon grant n. **GGP04113** is acknowledged.

NON STEROIDAL ANTI INFLAMMATORY THERAPY IN MDX MICE.

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Duchenne muscular dystrophy (DMD) is a lethal recessive X-linked muscular disease. The only beneficial pharmacological treatment is the use of corticosteroids but their mechanism of action is unknown. Corticosteroids exert anti-inflammatory effects but very few data are available to assess whether their anti-inflammatory activity is the explanation of their efficacy in the DMD treatment. The aim of this research is to compare the effect of corticosteroid and NSAID (Non Steroidal AntiInflammatory Drugs) administration in the mdx mouse model and therefore to test the relevance of inflammation control in DMD treatment. Mdx mice were treated with: methylprednisolone (MP) 1mg/kg/day, aspirin (ASA) 25 mg/kg/day, parecoxib (PC) 0.5 mg/kg/day. The extent of inflammation, necrosis and the newly formed fibers were evaluated in tibialis anterior muscle samples obtained from treated and untreated mdx mice of 30 d old. Inflammatory cells were conspicuous in mdx mice and extensive areas of infiltration were present. The treatment of mice with MP reduced the inflammation

area comparable to both NSAID drugs. The same result was obtained evaluating the area occupied by necrotic and degenerating myofibers in treated compared to untreated mdx mice. MP and both NSAIDs treatments were highly effective. The percentage of regenerating fibers, however, was not significantly different in untreated and treated mdx animals. The presence in treated animals of less degenerating fibers suggest that, overall, there could be a more sustained regeneration in treated mice. The same result was obtained with the analysis of mdx mice of 11 w old. Force measurements in vivo (gastrocnemius) and in vitro (diaphragm strips) did not show any significant difference between MP treatment and NSAID administration. These data suggest that chronic treatment with NSAIDs have a potential benefit on skeletal muscle morphology of mdx mice comparable with the effects of the treatment with the corticosteroid.

HISTOPATHOLOGICAL AND MORPHOMETRIC ANALYSIS HELP TO DIFFERENTIATE MYOTONIC DYSTROPHY TYPE 1 AND TYPE 2

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Myotonic dystrophy (DM) has been characterized on genetic ground through the association with two different loci identifying DM type 1 (DM1) and DM type 2 (DM2). Molecular analysis for genotyping DM2 is presently available only in a few centers. Since the clinical features of DM1 and DM2 may present striking similarity, muscle biopsy studies might be needed for differential diagnosis. Although the histopathological changes of DM2 muscles have been generally described as relatively mild and non-specific, detailed studies are lacking. Therefore, we performed a morphological and morphometric analysis on muscle biopsies from 11 DM1 and 13 DM2 patients. In DM1 cases, we observed preferential type 1 fiber atrophy, as previously described, and a higher prevalence of central nucleation among type 1 fibers in all cases. In DM2 muscle biopsies, atrophy of type 1 fibers was absent, while a high rate of hypertrophic fibers of either type was detected in most cases. Moreover, as opposed to DM1, all cases showed preferential central nucleation in type 2 fibers. These data contribute to better define specific histopathological patterns for DM1 and DM2, and may help clinicians to differentiate between these two forms of myotonic dystrophy and to correctly address genetic studies.

MYOGENIC EVENTS IN MUSCLE MASSIVE CRUSHING.

D.Biral, Alessandro Salviati, Vincenzo Vindigni, Franco Bassetto, Francesco Mazzoleni, Nicoletta Adami, Ugo Carraro

To study the regeneration of the muscle occuring in the tissue reconstruction after severe damage of the muscle we performed two types of experiments in the Rectus Abdominis muscle of the rat.

1) Crushing of the muscle, injection of marcaine, autograft after 3 days with or without concomitantly injection of marcaine and dissection of the muscle at about 3 weeks after operation.
2) Crushing of muscle, treatment of the muscle with marcaine immediately after crushing and after 3 days and dissection after 1 week and 3 weeks of operation.

We studied the operated muscle staining the Rectus Abdominis with ematoxylin and eosin, with myosin ATPase and with anti-embryonic myosin to valuate the degree of recovery. Percentages of the patch area covered by regenerated myofibers were determined by morphometry. The spontaneous regenerative process is about 10% of the area of autograft when we do not inject marcaine following autograft and is of about 20% in the presence of marcaine after autograft. The experiments made using crushing and marcaine demonstrate a recovery of about 16% after 1 week of operaration. 3 weeks after the operation the muscle is composed of mature fibers and only a small part of the muscle is composed of interstitial tissue. The only tratment of the Rectus Abdominis with marcaine demonstrate a complete recovery of the muscle after 3 weeks .

Cell migration during remodeling of the autograft involves a direct cellular response to chemoactractant factors. The formation of the blood clot after autograft composed of cross-linked fibrin and fibronectin serves as a matrix for invading cells and a reservoir of growth factors and cytokines. Myosin regeneration seems to be the results of successive waves of migration of angioblasts from the muscle surrounding the patch and is directely dependent from the angiogenesis and reinnervation of the muscle graft.

ADDENDUM

BPV REPROGRAMS MYOBLASTS TOWARDS THE PHENOTYPE OF A PLURIPOTENT, CIRCULATING PRECURSOR.

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Satellite cells are the main source of myogenic progenitors in post-natal skeletal muscle, but their use in cell therapy for muscle disorders is limited because these cells cannot be delivered through circulation and they are rapidly exhausted in severe myopathies. The search for alternative donor cells is ongoing but none of the candidates so far shows all the features required for successful colonization and repair of diseased muscle (1-3).

In this study we show that Bisperoxovanadium (BpV), a phospho-tyrosine phosphatase (PTP) inhibitor, induces satellite cells to acquire a gene expression profile and a differentiation potential consistent with the phenotype of circulating precursors, while maintaining their myogenic potential. These effects are mediated, at least in part, by NFkB activation through the Tyr42-IkB-□ phosphorylation, as shown by the expression of the dominant negative mutant form of the p50 NFkB subunit .

Moreover, when BpV-treated cells are injected into the femoral artery of □-sarcoglican null dystrophic mice, they are able to circulate and to reach muscle tissue; importantly, they contribute to muscle regeneration, as shown by the expression of □-sarcoglican in some fibers.

Our observations indicate that BpV, or similar compounds, may prove very valuable to obtain and to expand, from committed cells, multi-potent cell populations suitable for gene-cell therapy applications, and may help to understand the molecular basis of genome reprogramming.

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ATTENUATING SKELETAL MUSCLE WASTING IN CANCER-ASSOCIATED CACHEXIA

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Cachexia is a muscle wasting syndrome characterized by the loss of both skeletal muscle and adipose tissue, associated with chronic diseases such as AIDS and cancer. The specific mechanisms that underlie this muscle wasting are unknown, but recent evidence points to a role for proinflammatory cytokines and various proteolitic pathways including the ubiquitin-proteosome pathways.

IGF-1 has been implicated in many anabolic pathways in skeletal muscle; it promotes the activation of satellite cells, enabling them to repair damaged fibers. Mitogenic action of IGF-1 is mediated by activation of the MAPK pathway, whereas the differentiative effect is mediated by PI3-kinase pathway.

We plan to extend preliminary results, showing that conditioned medium (C.M.), obtained from C26 tumor cells, interferes with terminal muscle differentiation, whereas the post-mitotic expression of mIGF-1 counteracted the inhibitory effect of conditioned medium on muscle differentiation.

To this end we will use two myogenic cell line: C2C12 and L6MLC/mIGF-1.

Preliminary data showed that hrIGF-1 treatment enabled C2C12 to partially bypass the inhibitory effect of C.M. on skeletal muscle differentiation and there is no evidence of interference in the mechanism of differentiation in the case of L6MLC/mIGF-1 treated with C.M.

We verified whether local expression of mIGF-1 counteracts muscle decline in mouse.

To this purpose wild type and MLC/mIGF-1 transgenic mice were injected with murine C26 adenocarcinoma cells; this tumor causes significant loss of mass in muscles without significant effects on food intake (Tanaka et al., 1990).Histological analysis revealed that transgenic mice, transplated with tumor C26 cell line, presented muscle fibers larger than those of wild-type, injected with same tumor.

These preliminary data suggest that manipulation of mIGF-1 expression may be a useful tool to design specific therapeutic approach to target cancer cachexia.
Keywords: muscle wasting, mIGF-1, phenotype mainteniance.

INCOMPLETE MATURATION OF THE E-C COUPLING APPARATUS IN MICE LACKING CALSEQUESTRIN-1.

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Ca^{2+} plays an important role in many cellular functions, from gene expression, to cell differentiation, etc. Along these lines, there is increasing evidence indicating that the EC coupling apparatus, a specialized system of membranes that finely controls the release/uptake of Ca^{2+} from the SR in muscle, has a central role in the correct execution of the differentiation and maturation programs. The major SR Ca^{2+} binding protein, calsequestrin (CS), is expressed in two isoforms in skeletal muscle. CS2, abundant in fetal and neonatal stages, in fast-twitch fibers disappears completely in the first few weeks after birth. Meanwhile, the accumulation of CS1 progressively increases to adult levels, in the same critical period in which profound structural and molecular modifications transform fibers from neonatal into mature. To identify a possible role of CS1 in the full maturation of fast-twitch fibers, we characterized EDL muscles from 7d to 4m of age in a knock-out model *null* for CS1. Western-blot analysis indicates that CS2 expression does decrease in the first postnatal months, just as in WT EDL leaving the majority of fibers without any CS. Structural maturation of CS1-*null* skeletal muscle fibers, studied using a combination of thin-sectioning and T-tubule staining for EM, is characterized by an incomplete reorganization of the T-tubular system from longitudinal to transversally oriented, as it would normally occur in WT muscle. Also frequency of longitudinal and multi-layered junctions, typical of early developmental stages, is significantly higher. Furthermore, the time parameters of the twitch are prolonged making the adult CS1-*null* EDL more similar to the slow Soleus or to immature EDL muscle. These findings suggests a crucial role of CS1 in the correct execution of the differentiation and maturation programs of the EC coupling apparatus in fast-twitch fibers.

AGEING EFFECTS IN HUMAN MUSCLE SATELLITE CELLS: MODULATION OF TRANSCRIPTIONAL PROFILE IN RELATION TO FUNCTIONAL MODIFICATIONS.

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Sarcopenia is an age-related condition characterised by a progressive loss of mass and strength in skeletal muscle (Exp.Gerontol.39:17,2004). In our previous works we showed a decreased activity in the antioxidant system in skeletal muscles, myoblats and myotubes derived from sarcopenic aged individuals, probably due to an abnormal accumulation of free radicals (Free Rad.Biol.Med.26:303,1999; Exp.Gerontol.40:189,2005). One of the main question still to be answered is why the satellite cells on aged muscle are unable to regenerate new functional fibers counteracting the sarcopenic process. We studied the transcriptional profile of undifferentiated and differentiated (4, 24 and 72 h) cells in order to understand if there is an oxidative damage-alteration of signalling-pathways on senescent myoblasts and myotubes with an impairment of their differentiative potential. We found up-regulated in myoblasts Polymerase kappa that suggests an attempt to the DNA repairing. Moreover, we found up-regulation of genes involved in proteasome pathway. Indeed, atrophic myotubes were observed in the cultures from aged donors. It is worth mentioning also many genes relevant to actin cytoskeleton regulation and extracellular-matrix-receptor interaction and in particular a strong up-regulation of gene encoding collagen, proteoglycan, laminin isoforms. The lipid peroxidation influences both plasmamembrane and extracellular matrix functionality. We also found a probably alteration of excitation-contraction coupling

mechanism during the ageing process. With these data we hypothise that sarcopenia involves satellite cells as well and determine impairment in their differentiative program.

SYSTEMIC DELIVERY OF AAV VECTOR EXPRESSING ANTISENSE-U1 SNRNA: EFFECTS OF DIFFERENT VIRAL DOSES ON DYSTROPHIN EXPRESSION AND FIBRES FUNCTION IN SKELETAL MUSCLES OF *MDX* MICE.

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Mutations in the dystrophin gene, causing Duchenne muscular dystrophy, can be corrected at the post-transcriptional level by skipping the mutated exon in the mature mRNA. Recently we demonstrated persistent exon skipping in *mdx* mice by systemic delivery of an adeno-associated viral (AAV) vector expressing antisense sequences as part of the stable cellular U1 small nuclear RNA against exon 23. We injected *mdx* mice with different doses of the construct AAV-U1#23 (10^8, 10^{10} and 10^{12} U/L) to study expression and function of reconstituted dystrophin and evaluate the minimal viral dose able to support a functional recovery.
Muscle function was evaluated *in vitro* by measuring specific force (Po/CSA) of fibre segments from the gastrocnemius and tetanic force of intact preparations of the diaphragm and *in vivo* by treadmill exhaustion tests. Single fibres were characterized for myosin heavy chain content and dystrophin expression. A significant recovery of specific force (Po/CSA) was observed in dystrophin positive vs. dystrophin negative fibres. Diaphragm preparations also showed a significant increase of normalized tetanic force (Po/V) after treatment and the functional recovery of treated mice was confirmed by treadmill exhaustion tests. We conclude that exon skipping of the mutated exon 23 through systemic delivery of 10^8U/L AAV/antisense-U1 induces the formation of a working dystrophin sufficiently to promote functional recovery of *mdx* mice.

MEASUREMENT OF VISCOELASTIC PROPERTIES OF MLC/mIgf-1 DISTAL TENDON FROM EDL, SOLEUS AND TIBIALIS ANTERIOR MUSCLES.

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MLC/mIgf-1 transgenic mice are a model of persistent, functional hypertrophy, in which fast skeletal muscles present a significant increase in tetanic force and in maximum mechanical power.
In this work we investigated the viscoelastic properties of distal tendon from MLC/mIgf-1 EDL, Soleus and Tibialis Anterior of both wild type and transgenic mice. This analysis allowed us to evaluate if the muscle expression of mIgf-1 presented paracrine effects on a tissue not directly involved in the overexpression of the transgene, but firmly connected to hypertrophic muscle tissue.
Complex compliance is a parameter frequently employed to study the viscoelastic behaviour of soft tissues; it is made up of two mathematically orthogonal components: the storage compliance, which reflects the elastic component of the tissue, and the loss compliance, which is related to the viscous behaviour of the tissue.

The experimental protocol led to the calculation of tissue complex compliance for seven different frequencies using a first-order Volterra-Wiener constitutive model, calculated stimulating the specimen with a Pseudorandom Gaussian white Noise stress input signal.
A two way ANOVA analysis was applied separately to storage compliance (SC), loss compliance (LC) and phase angle (PA) considering mouse type and frequency as fixed factors.
Experimental results performed for SC and LC showed no differences in the viscoelastic properties of EDL tendons of both wild type and MLC/mIgf-1 transgenic mice. On the contrary, Tibialis Anterior of MLC/mIgf-1 tendons showed lower values of SC for each frequency of interest, while LC didn't differ between the two models.
Finally tendons from transgenic Soleus showed lower values of SC, LC and PA for each frequency.
In conclusion overexpression of mIgf-1 seems to present paracrine effects on tendinous tissue, in particular on tendon of Soleus muscle: these tendons present an increased stiffness and a decreased viscosity, resulting in a lower propensity to inflammation.