SESSION I

MUSCLE DISEASES AND THERAPIES I

FINAL REPORT OF THE EUROPEAN PROJECT "RISE".

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Spinal-cord injury causes loss of function and muscle atrophy, which is especially severe when lower motor neurons (LMN) are involved. A longitudinal study in 25 Europeans suffering of complete Conus Cauda Syndrome from 0.7 to 8.7 years compared thigh muscle properties before and after two years of home-based daily Functional Electrical Stimulation (FES). Muscles were stimulated by large surface electrodes and a custom-designed stimulator. Muscle poor excitability was first improved by twitch contraction training. Tetanic contractions without and, later on, with increasing load were then elicited. Finally, standing-up exercises were daily performed. The bulk of thigh muscles were estimated by transverse CT scan and force measurements. Needle muscle biopsies were harvested before and after two years of FES and analyzed by light microscopy. Two years of home-based daily FES: 1. Induced similar muscle recovery in both legs, as shown by CT scan (+35%), biopsy morphometry (+76%, and the comparison of fiber size profiles shows that this is the result of a shift toward larger-size muscle fibers) and force measurements (+ 900%); 2. Improved cosmetic appearance and muscle cushioning effect of thighs; 3. Despite the additional two years of denervation, none of 20 compliant subjects worsened, while 5 (25%) improved up to allow standing-up. The EU Project Rise demonstrates that FES is an effective home-based therapy that may maintain life-long motivation to perform *active* exercise (electrical stimulation is the only option for denervated muscle) and training of leg vascular bed as an adjuvant measure to *decubitus* prophylaxis.

MOLECULAR MECHANISMS REGULATING SKELETAL MUSCLE HOMEOSTASIS: EFFECTS OF V1a AVP RECEPTOR OVER-EXPRESSION.

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The maintenance of a working skeletal musculature is conferred by its remarkable ability to regenerate after pathological or mechanical damage. However most muscle pathologies, such as cachexia, are characterized by the progressive loss of muscle tissue due to alterations of healthy skeletal muscle homeostasis. Arg-vasopressin (AVP) is a potent myogenesis promoting factor known to induce myogenic differentiation and hypertrophy *in vitro.*
Here we demonstrate that local over-expression of V1a AVP receptor (V1aR) in injured muscle results in: 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 an animal model of cachexia, induced by elevated TNF-□ levels in muscle, V1aR over-expression is able to counteract the negative effects of cachexia. In this condition, V1aR over-expression results in increased Pax-7, myogenin and myosin expression levels. The positive effects of V1aR on muscle homeostasis are due to the stimulation of the calcineurin-IL-4 pathway and to the inhibition of atrophic genes expression, mediated by FOXO phosphorylation.
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.

THE BLOCK OF CA-DEPENDENT K^+CHANNELS REDUCES MYOTONIA IN STEINERT DISEASE: AN *IN VIVO* PHARMACOLOGICAL STUDY.

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Many studies have been carried out to clarify the mechanism underlying the abnormalities of sarcolemma in Myotonic Dystrophy type1 (MD) but univocal results have not been reported.
The first clinical evidence of specific channels involvement was suggested after the observation that the local treatment of muscle with apamin, a specific blocker of Ca-activated K^+-channels (SK), reduced the "myotonic runs" in MD (Beherens et al.,1994). Recently we showed a characteristic surface EMG pattern, strictly leaked to myotonia, in MD patients (Chisari et al.,2001).
In this study we evaluated the effect of the local administration of apamin on sarcolemma excitability alteration recorded through surface and needle EMG.
We applied a stimulation protocol to 8 MD patients and recorded an amplitude parameter (ARV) of surface EMG before and after the local injection of 50 μl of 10 μM apamin. Moreover, to verify the reliability of our approach, in two patients we recorded the needle EMG "myotonic runs" before and after the local injection of apamin.
According to Beherens et al., we observed a clear reduction of myotonic discharge recorded by means of needle EMG. On the other hand, in 2 out of 8 patients we observed a complete but transient normalization of the characteristic surface EMG pattern.
This work confirmed the role of SK in sarcolemma "instability" represented by the needle EMG "myotonic runs" and did not rule out the hypothesis that SK could play a specific role in the genesis of phenotypic expression of myotonia in MD. Of course further studies need to validate this hypothesis but we consider this approach a good starting-point to study ***in vivo*** muscle functional alteration in Myotonic Dystrophy type1.

RyR1-Y522S/+ MICE: A NEW ANIMAL MODEL TO STUDY CORES MYOPATHIES IN SKELETAL MUSCLE.

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Malignant hyperthermia (MH) and central core disease (CCD) are closely related diseases of skeletal muscle linked to mutations in the ryanodine receptor (RyR1) gene. Heterozygous mice harbouring a human MH mutation (Y522S), which is linked MH susceptibility (MHS) with cores, were shown to display a typical MH phenotype (Durham et al., 2008; Cell: 133, 53). Here we show that $RyR1^{Y522S/wt}$ fibers develop *cores* which mimic, in the later stages, the structural alterations described in several human examples of CCD. By examining mice at various ages (2 m - 1 y), we identified crucial steps in the formation of cores, a feat that has never been possible in the humans. The earliest and most obvious event in the formation of "core regions" is the swelling and partial disruption of mitochondria. This defect quickly leads to disarray of closely associated structures, such as sarcoplasmic reticulum (SR) and calcium release units (CRUs), and eventually causes complete disorganization and dissolution of both organelles in confined areas. Once SR, CRUs, and mitochondria are absent, the "core regions" lose their ability to efficiently re-uptake the Ca^{2+}, which diffuses from neighbouring areas. Prolonged accumulation of Ca^{2+} in restricted areas causes, first, prolonged contractures and, second, hydrolysis of the contractile material and formation of amorphous cores. Contracted and amorphous cores closely mimic the cores described in humans.

STEM CELL HOMING AND REGULATION: NEW INSIGHTS FROM MUSCULAR DYSTROPHY.

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In muscular dystrophies a cardiac involvement represents a complication leading the patients to a more dramatic worsening of health condition. Limb-girdle muscular dystrophy type 2E (LGMD 2E) is caused by mutations in the *sarcoglycan* gene. The expression of the sarcoglycan complex is necessary for the stabilization of dystrophin at the sarcolemma level: depletion of this complex results in degeneration of skeletal and cardiac myofibres. Recently, we showed that a class of vessel associated stem cells, named *mesoangioblasts*, isolated from muscle biopsies of mice, dogs and humans [1] can regenerate skeletal muscles in dystrophic subjects. Moreover, wild-type cardiac mesoangioblasts can efficiently differentiate *in vitro* an *in vivo* into cardiomyocytes. We isolated and characterized cardiac mesoangioblasts (cdMABs) from heart biopsies of a dystrophic mouse model of LGMD 2E. In particular, we characterized the expression of surface markers and transcription factors and we quantified the ability to migrate into heart and filter organs, after intravenous delivery into cardiomyopathic mice. Despite their ability to home and differentiate into cardiomyocytes, cdMABs fail to regenerate necrotic areas of the myocardium. By real time expression array, their transmigration and transdifferentiation ability seems affected by signal-transduction pathways, such as *Notch* and *Wnt* signalling, that drive cell fate. Exploring the balancing patterns of these molecular signals will probably shed new light on myogenic program and dystrophy therapy.

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SESSION II

MUSCLE DISEASES AND THERAPIES II

MIGF-1 MODULATES CASPASE AND CDK5 EXPRESSION IN SKELETAL MUSCLE OF A MOUSE MODEL OF ALS.

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The functional connection between muscle and nerve is often altered in several neuromuscular diseases, including amyotrophic lateral sclerosis (ALS). Knowledge about the molecular and cellular mechanisms involved in the restorative reactions is important to our understanding of the processes involved in neuromuscular maintenance. We previously reported that muscle-restricted expression of a localized Igf-1 isoform maintained muscle integrity, stabilized neuromuscular junctions, reduced inflammation in the spinal cord and enhanced motor neuronal survival in SOD(G93A) mice, delaying the onset and progression of the disease. In this study, we analysed potential molecular pathways that are modulated by mIgf-1 to counteract muscle wasting and to preserve motor neurons activity. We performed molecular and morphologic analysis to address the specific proposed questions.. Ubiquitin expression and caspase activity resulted markedly increased in SOD(G93A) muscle but maintained at very low levels in the SOD(G93A) x MLC/mIgf-1 (SOD(G93A)/mIgf-1) transgenic muscle. In addition, CDK5 expression, a serine-threonine protein kinase that has been implicated in a number of physiologic processes in nerve and muscle cells, was reduced in SOD(G93A) muscle but increased in SOD(G93A)/mIgf-1 muscle. Notably, while the toxic p25 protein accumulated in SOD(G93A) muscle, no accumulation was evident in the SOD(G93A)/mIgf-1 muscle. The maintenance of muscle phenotype was also associated with maintenance of a normal peripheral nerve, and a greater number of myelinated axons. These observations offer novel insights into the role of mIgf-1 in the attenuation of muscle wasting in the mouse model of ALS disease.

CDK9-55: A NEW PLAYER IN MUSCLE REGENERATION.

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Adult skeletal muscle contains a specialized population of myogenic quiescent stem cells, termed satellite cells, which contribute to repair myofibers after injury. During muscle regeneration, satellite cells exit their normal quiescent state, proliferate, activating MyoD and Myf-5 expression, and finally differentiate and fuse to reconstitute the injured muscle architecture. We have previously reported that cdk9 is required for myogenesis *in vitro* by activating MyoD-dependent transcription. In myoblasts induced to differentiate, MyoD recruits cdk9 on the chromatin of muscle-specific regulatory regions. This event correlates with chromatin-modifying enzyme recruitment and phosphorylation of cdk9-specific target residues at the carboxyl-terminal domain of RNA polymerase II. Here we report that a second cdk9 isoform, termed cdk9-55, plays a fundamental role in muscle regeneration and differentiation *in vivo*. This alternative form is specifically induced in injured myofibers and its activity is strictly required for the completion of muscle regeneration process.

CARDIAC MESOANGIOBLASTS ARE COMMITTED, SELF-RENEWABLE PROGENITORS, ASSOCIATED WITH SMALL VESSELS OF BOTH ADULT NORMAL AND CARDIOMYOPATHIC MOUSE VENTRICLE.

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Different cardiac stem/progenitor cells have been recently identified in the post-natal heart (1,2). We describe the identification, clonal expansion and characterization of self-renewing progenitors that differ from all those previously described for high spontaneous cardiac differentiation. Unique co-expression of endothelial and pericyte markers identify these cells as cardiac mesoangioblasts (Mabs) and allow prospective isolation and clonal expansion from the adult mouse ventricle. Cardiac Mabs express many cardiac transcription factors and spontaneously differentiate into beating cardiomyocytes that assemble mature sarcomeres and express cardiac ion channels. When injected into the ventricle after coronary artery ligation, cardiac mesoangioblasts efficiently generate new myocardium in the border of the necrotic zone.
Based upon these results we also demonstrate that cardiac mesoangioblasts could be isolated from hearts of beta sarcoglycan (SG) null mice affected by cardomyopathy but they may fail in the myocardium repairing because of an impairment in their differentiation process. In particular we show that atrium and ventricle clones express both cardiac early markers, such as Nkx2.5, GATA 4 and Isl1 and muscle specific trascription factors, such as MyoD and Myf5. It's our interest to investigate a possibile signalling activity of beta SG protein in muscular differentiation process.
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IN VIVO DELIVERY OF NAKED AND LIPID-COMPLEXED ANTISENSE OLIGOS IN MDX MICE: EFFECTS ON SKELETAL AND CARDIAC MUSCLE.

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Antisense-mediated exon skipping holds great potential for the treatment of DMD. In mdx mice, functional recovery of skeletal muscle has been reported upon systemic delivery of "naked" oligonucleotides or viral vectors encoding for antisense snRNAs.
However, only one study achieved dystrophin restoration in cardiac muscle (using an adeno-associated vector). Here we report the in vivo delivery of morpholino oligos in aged mdx mice, both in skeletal muscle, via intra-arterial injection, and in cardiac muscle, via intramuscular injection. Intra-arterial delivery yielded levels of dystrophin restoration comparable to those reported in the literature with the intra-venous approach, but with smaller amounts of oligonucleotides. Intra-cardiac injections, on the other hand, showed that the level and duration of the skipping effect found in cardiac muscle were greatly decreased compared to skeletal muscle. This latter finding provides the first direct evidence that antisense-mediated dystrophin restoration in cardiac muscle might suffers from limitations that do not exist in skeletal muscle.
All data published so far have indicated that systemic delivery via the vasculature requires large amount of naked oligos to achieve therapeutically significant results. Here we also report that the use of lipid carriers has the potential to greatly improve the delivery efficiency; in particular, we found that the use of lipid-encapsulated oligo RNA allowed to detect dystrophin re-expression with a single dose of ~40 μg of oligos per adult mdx mouse. Importantly, dystrophin restoration could be seen not only in skeletal and but also (albeit to a smaller extent) in cardiac muscle.

ANTIOXIDANT TREATMENT OF MOUSE *IN VIVO* DOES NOT PREVENT HINDLIMB UNLOADING-INDUCED MUSCLE ATROPHY.

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Oxidative stress has been observed in various conditions of muscle atrophy, but it is not known if it is a cause or consequence of muscle wasting. The rodent hindlimb unloading (HU) is a model of severe atrophy. In this study, 6 month-old mice were hindlimb-unloaded for 14 days (HU14) and the properties of slow-twitch soleus (Sol) and fast-twitch gastrocnemius (Gas) muscles were studied. A number of animals received daily trolox injections (HUTRO) one week prior HU and during the two weeks of HU to verify if a potent and selective antioxidant may be an efficient countermeasure against disuse-induced muscle atrophy. Muscle-to-body weight ratio indicated significant atrophy in Gas and Sol after HU14, not prevented by trolox. A reduction of the cross-sectional area (CSA) was observed in both MHC-1 and MHC-2A positive fibres of HU14 Sol muscle. The MHC isoform distribution in Sol muscle changed towards a faster phenotype after HU14 but less in HUTRO mice, indicating that trolox prevented partially the shift of Sol muscle phenotype. After HU14, the resting chloride conductance of sarcolemma (gCl) increased significantly in Sol and Gas muscle fibres. Remarkably, HUTRO mice did not show any change in the gCl of both Sol and Gas muscle fibres with respect to CTRL mice, indicating that trolox fully prevented the disuse-associated change in gCl. RestCa decreased in Sol muscle after HU14 but remained unchanged in the fast Gas muscle. Trolox reduced restCa in both muscles. The Sol and Gas muscle levels of malondialdehyde (MDA) and reduced glutathione (GSH), indicators of oxidative stress, were increased and decreased, respectively, in HU mice but not HUTRO mice. In conclusion, the prevention of oxidative stress by trolox was able to prevent, at least partially, the changes in MHC distribution and gCl, but did not prevent restCa changes and atrophy, suggesting that in muscle disuse, oxidative stress is rather a consequence than a cause of muscle atrophy. (Supported by ASI-OSMA).

SESSION III

CELL BIOLOGY AND MYOGENESIS

GENE EXPRESSION PROFILE OF GTP-DEPENDENT DIFFERENTIATION OF C2C12.

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In our previous studies we characterized the effect of extracellular guanosine 5' triphosphate (GTP) on Ca^{2+} homeostasis of mouse skeletal muscle cell line C2C12 via specific binding sites for GTP on plasma-membrane (Pietrangelo et al., *JMRCM*, 2002). 500 μM GTP is able to increase $[Ca^{2+}]_i$ and hyperpolarize myoblasts via intermediate Ca^{2+}-activated K^+ channels (Pietrangelo et al., *J. Physiol.*, 2006).
In the present study we focus our attention on the modulation of gene expression profile (using microarray and Real Time-PCR techniques) induced by 500 μM GTP on the C2C12 differentiation process.
Our data indicate that cells differentiated for 4 and 24 h in a synthetic media (Dulbecco's modified Eagle medium with 1% of bovine serum albumin) and in presence of 500 μM GTP show an increased expression of *Pax7* and *MyoD*, genes involved in myogenic regulation, *ncam-180* and *v-cam*, genes involved in cellular adhesion; *nectidin* that inhibits cell proliferation interacting with apoptotic factor E2F1; *calreticulin*, a multifunctional protein that acts as a major Ca^{2+}-binding protein in the lumen of the endoplasmic reticulum; *MARCKS*, a substrate of PKC, important for cellular adhesion and reorganization of cytoskeletal actin; *GSK-3*, a protein serin kinase that modulate trascription of NF-kB (Saa3); genes for enzymes like *cox7b*, *cox7a1*, *gapd* and mitochondrial enzymes.
Using Real Time-PCR technique we confirm the up-regulation of *GSK3β*, *Pax7* and *calcineurin* after 24 h of differentiation in GTP-stimulated C2C12 myotubes.
Considering the data here presented, we suggest that the specific GTP-binding sites on C2C12 cell membranes is capable to accelerate the myogenesis via modulation of the gene expression pattern.

SPHINGOSINE KINASE/SPHINGOSINE 1-PHOSPHATE AXIS IS IMPLICATED IN THE TRANSFORMING GROWTH FACTOR-β-DEPENDENT MYOFIBROBLASTIC DIFFERENTIATION OF MYOBLASTS.

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The pleiotropic cytokine transforming growth factor-β (TGFβ) has been reported to impair myoblast differentiation during myogenesis (1) and favour muscle fibrogenesis (2). Although it has been demonstrated that the canonical SMAD-dependent signalling cascade is implicated, the precise signalling pathways downstream of TGFβ accounting for its biological activity are largely unknown.
Recent studies have established the occurrence of a direct functional interaction between the TGFβ- and sphingosine 1-phosphate- (S1P) regulated signalling pathways. Since we have already provided evidence for a role of sphingosine kinase (SK)/S1P axis in myoblast differentiation (3), here to gain insights into the mechanism of action of TGFβ in myoblasts, the effect of the cytokine on SphK has been examined together with its possible implication in the evoked biological response.
Our results show that TGFβ exerts a biphasic effect on SK: the enzymatic activity was indeed inhibited within the first 4 h of incubation whereas it was enhanced at more prolonged times of incubation (18-72 h). The enhancement of SK activity was accompanied by the up-regulation of SK1, indicating that TGFβ exerts a transcriptional control of SK1. Interestingly, the pro-fibrotic effect of TGFβ, monitored by detection of α-smooth muscle actin expression, was attenuated when SphK1 was pharmacologically inhibited or down-regulated by specific short interfering RNA. Moreover, by employing selective agonists and antagonists for S1P receptors, as well as RNA interference technology, it was shown that the TGFβ-regulated expression of fibrosis marker was downstream of S1P3. These data demonstrate that SphK/S1P axis is exploited by TGFβ to transform skeletal myoblasts into myofibroblasts.

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BISPEROXOVANADIUM, A PHOSPHO-TYROSINE PHOSPHATASE INHIBITOR, REPROGRAMS MYOGENIC CELLS TO ACQUIRE A PLURIPOTENT, CIRCULATING PHENOTYPE.

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Satellite cells are considered to be the main source of myogenic progenitors in post-natal skeletal muscle. However, 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. We have recently shown that Bisperoxovanadium (BpV), a phospho-tyrosine phosphatase inhibitor, induces myogenic cells (both C2C12 and muscle primary cells) to acquire a gene expression profile and a differentiation potential consistent with the phenotype of a circulating precursor, while maintaining their myogenic potential. Moreover, when BpV-treated C2C12 cells are injected into the femoral artery of α-sarcoglycan null dystrophic mice, they are able to circulate and reach muscle tissue; importantly, they contribute to muscle repair, as shown by the expression of α-sarcoglican in some fibers (Castaldi et al. *Faseb J.*, 21: 3573-3583, 2007).

Whether a similar gene expression profile and behaviour is activated in all the BpV-exposed cells, or whether different cell sub-populations arise upon BpV treatment, is not clear. To address this question, we are currently comparing gene expression profiles following BpV treatment of freshly FACS-purified satellite cells vs. PICs. PICs are PW1-expressing interstitial cells, a newly described source of myogenic progenitors. Results of this screen are expected to identify the molecular players responsible for BpV's de-differentiating effect on myoblasts and the effect of BpV-induced signaling on muscle cell phenotype. These and other ongoing preliminary data will be discussed.

DIFFERENTIAL ENGAGEMENT OF RAGE AND FGFR1 IN MUSCLE SATELLITE CELLS BY S100B PROTEIN: INVOLVEMENT OF RAGE IN SATELLITE CELL ACTIVATION AND OF FGFR1 IN SATELLITE CELL EXPANSION.

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S100B inhibits myoblast differentiation and apoptosis and stimulates myoblast proliferation via activation of the mitogenic MEK-ERK1/2 and inhibition of the promyogenic p38 MAPK (1,2). However, a short-term (24 h), but not long-term treatment of low-density (LD) myoblasts with S100B resulted in enhanced myogenic differentiation via the simultaneous activation of p38 and ERK1/2. These differential effects of S100B depended on engagement of RAGE (receptor for advanced glycation end products) in LD myoblasts and of bFGF receptor (FGFR) 1 in high-density myoblasts. We show that intramuscular injection of S100B causes activation of muscle satellite cells (SCs) and stimulation of their proliferation, as inferred by the increased numbers of Pax7-positive cells and BrdU-positive cells, respectively, compared with sham-injected muscles. By contrast, no such effects can be observed upon injection of S100B into muscles of RAGE null mice. S100B is expressed in mature myofibers and is present in crushed muscle extract (CME). Blocking S100B in CME with an S100B neutralizing antibody results in reduced ability of CME to stimulate myoblast proliferation. Also, a short-term (24 h) treatment of LD myoblast cultures with S100B on day 4 after their switch to DM results in enhanced myotube formation and myotube hypertrophy likely due to S100B-induced activation of quiescent myoblasts and stimulation of their proliferation. Indeed, S100B accelerates the proliferation of myoblasts upon their switch from quiescence medium to DM. Collectively, our results suggest that S100B released from damaged muscles might activate SCs via RAGE engagement. However, S100B enhances the proliferation of RAGE null primary myoblasts, an effect that is blunted in the presence of an FGFR1 neutralizing antibody. Thus, S100B might activate muscle SCs via RAGE engagement and expand activated SCs via FGFR1 engagement.

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EXPRESSION OF THE GAP JUNCTION PROTEIN CONNEXIN43 REGULATES SPHINGOLIPID METABOLISM IN SKELETAL MUSCLE CELLS.

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Although much remains to be learned on the molecular mechanisms involved in the myogenesis, it is well established that diverse intercellular signaling pathways may influence the regulation of myoblast differentiation and regeneration of skeletal muscle. One of them is mediated by gap junctions, specialized membrane regions formed by the conjunction of two hemichannels composed mainly by connexin (Cx) 43. Cx43 expression appears to be up-regulated during skeletal muscle regeneration and required for normal myogenesis *in vivo*. In agreement with these evidence, we recently shown that the regulation and assembly of connexins into gap junctions and Cx43 protein per se represent critical events in myoblast differentiation elicited by sphingosine 1-phosphate (S1P), a bioactive lipid implicated in this process and in the activation of muscle stem cells.

Here we investigated the possible cross-talk between Cx43 expression and function and sphingolipid metabolism in primary cultures of myoblasts obtained from wilt type and Cx43-knockout (Cx43KO) newborn mice. Interestingly, we found that sphingolipid metabolism is significantly altered in Cx43KO cells compared to wild type and $Cx43^{-/+}$ cells.

These findings suggest that the ability of the cells to share information and/or the signalling pathways mediated by Cx43 per se are required for the correct balance between sphingolipids, in particular ceramide and S1P, two messenger molecules that act in opposite manner in the coordination and guide of the interacting myoblasts to their final differentiation.

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THE ROLE OF NICOTINIC ACETYLCHOLINE RECEPTOR CLUSTERS IN CALCIUM SPIKING AND TWITCHING IN DIFFERENTIATING MOUSE SKELETAL MYOTUBES.

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During myogenesis, clusters of embryonic muscle nicotinic acetylcholine receptors (nAChRs) are present before the arrival of the motor neuron. Autocrine activation of nAChR contributes to the spontaneous oscillations of intracellular concentration of Ca^{2+} and to twitches characteristic of developing mouse myotubes before innervation. The present work was aimed at testing if the aggregation of the nAChRs could modulate the Ca^{2+} spiking activity during myogenesis.
Mouse myoblasts were differentiated *in vitro* up to 12 days. The distribution of the nAChRs was analysed by confocal microscopy. The Ca^{2+} spikes were measured by videoimaging and the single channel properties of nAChRs by patch clamp.
Clusters of nAChRs were observed starting from day 2 of differentiation. Their number was the highest after 11 days, when clusters were detected in 39.85 ± 2.59 % of the cells (1 cluster per cell; mean length 11.76 ± 0.50 mm). The nAChR labelling showed that 75.75 ± 3.87 % of the cells with clusters exhibited spiking and twitching. A 24 h incubation with STI 571 10 □M, a specific inhibitor of Abl (nonreceptor tyrosine kinases which control the nAChR clustering) significantly decreased the number of cells with clusters (to 13.39 ± 2.40 %). The pharmacological treatment decreased also the number of spiking and twitching cells (from 23.95 ± 1.81 % to 7.40 ± 0.91 %). Patch clamp recordings showed that STI 571 did not affect amplitude and kinetics of the nAChRs.
In conclusion, our results indicate that the formation of nAChR clusters could favour spontaneous Ca^{2+} spiking and twitching during myogenesis. These data suggest a possible new role for the so-called “prepatterned AChR clusters” before the arrival of the nerve.

SESSION IV

FROM THE ELECTRICAL IMPULSE TO THE SR Ca^{+2} RELEASE

AGRIN CHANGES THE ELECTRICAL PROPERTIES OF DEVELOPING HUMAN SKELETAL MUSCLE CELLS.

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Agrin is a heparan sulphate proteoglycan, known for its involvement in the organization and maintenance of postsynaptic structures at the neuromuscular junction (NMJ). Recent investigations suggest that the effects of neural agrin might not be only synaptogenic; other aspects of muscle development might be promoted by agrin.
To investigate the role of agrin in the modulation of the electrical properties of single muscle cells, we did whole-cell patch clamp recordings on cultured never innervated human myotubes derived from satellite cells. Changes in the negative direction of the resting membrane potential in the agrin-treated muscle cells were found. Such modifications were correlated to a higher contribution of the strophantidin-sensitive Na^+/K^+ ATPase in establishing the resting membrane potential and to quantitative modification in the amount of the α1 and α2 subunits of the Na^+/K^+ ATPase.
A down-regulation of the small-conductance (SK) K^+ channel activity has been reported during development after innervation; we also observed a significant decline of SK channel activity after agrin application in the culture medium.
In conclusion, our results show that, besides its well recognised synaptogenic effects in skeletal muscle, alternative effects can be induced by agrin also in muscle itself where they are not limited only to AChR clustering and NMJ formation, but extended also to other aspects of muscle differentiation.

IMPAIRED MUSCLE OXIDATIVE METABOLISM IN INFLAMMATORY MYOPATHIES EVALUATED *IN VIVO* THROUGH LACTIC ACIDAEMIA ASSAY.

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Polymyositis and Dermatomyositis (PM/DM) are inflammatory myopathies of unknown aetiology. Muscle weakness, myalgias and generalized fatigue lead to malaise and decreased use of striated muscles. Previous works showed impaired oxidative metabolism in PM and DM. Moreover some authors described a reduced muscle mitochondrial function.
In a PM/DM group we assessed the lactic acidaemia at rest and after an aerobic exercise, to test *in vivo* the muscle oxidative metabolism efficiency.
Method. A group of 14 PM/DM pts performed a submaximal incremental exercise on a treadmill. The exercise consisted of: 11 steps, each of 2' of duration, at a constant speed (3 Km/h) with a grade that was 0° at the beginning and increasing of 2.5% at each step. Lactic acidaemia was assessed at rest and after 1', 5', 10', and 30' from the end of exercise.
Results. Lactate at rest was not significantly different in two groups. At the end of exercise and during recovery PM/DM showed a significantly higher rise of lactate respect to controls. A decreased mean time of duration of exercise and a precocious fatiguability was observed. Finally, no correlation among disease activity, pharmacological treatment, physical activity and lactate was found.
Conclusion. The data obtained in this study can be referred to a **defective muscle oxidative metabolism** in patients with Polymyositis and Dermatomyositis.
This metabolic abnormality can play a role in the **excessive fatiguability** observed in these patients.
A **specific aerobic training** may be advisable for these patients to improve the muscle performance and to reduce the symptom fatigue.

EFFECTS OF ELOCALCITOL ON L-TYPE Ca^{2+} CHANNELS OF SMOOTH MUSCLE CELLS FROM HUMAN BLADDER.

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Elocalcitol is a Vitamin D receptor agonist known to inhibit RhoA/Rho kinase signalling in human bladder and has been demonstrated to improve the symptoms of overactive bladder, that is an uncoordinated myogenic activity. The smooth muscle contraction in human bladder mainly depends on two mechanisms: L-type Ca^{2+} channels activation and a Ca^{2+}-independent RhoA/Rho-kinase pathway. First of all, we observed that Elocalcitol (10^{-12}-10^{-7}M) was able to induce a dose-dependent increase in intracellular [Ca^{2+}] in smooth muscle bladder cells (hBCs), with maximal effect at 10^{-7}M. The pre-treatment with the specific L-type Ca^{2+} channel antagonist verapamil as well as the absence of extracellular Ca^{2+} abolished such Ca^{2+} transients, strongly suggesting an influx through L-type Ca^{2+} channels. Thus, we addressed our study to investigate the effects of Elocalcitol on L-type Ca^{2+} channels. Accordingly, we performed electrophysiological analysis by the whole-cell patch-clamp and we showed that our cells exhibited voltage-activated T-type and L-type Ca^{2+} currents ($I_{Ca,L}$). Isradipine and verapamil only blocked the slow $I_{Ca,L}$, which was enhanced by the selective L-type Ca^{2+} channel agonist Bay K8644. The adding of Elocalcitol (10^{-7} M) caused an increase in $I_{Ca,L}$ size and specific conductance (G_m/C_m), by inducing a faster activation and inactivation kinetics than control and Bay K8644, and determined a significant negative shift of the activation (V_a) and inactivation curves (V_h), as Bay K8644. In long-term treated (48h) hBCs Elo (10^{-8} M,) caused a further significant shift of V_a and V_h towards more negative potentials and a significant increase of both I_{Ca} amplitude and G_m/C_m, which were abolished by verapamil. In long-term-treated hBCs Elocalcitol was capable to induce mRNA and protein expression of pore forming L-Type α_{1C} subunit, as revealed by real time RT-PCR and western blot analysis. In conclusion, Elocalcitol was able to upregulate Ca^{2+} entry through L-type Ca^{2+} channels in hBCs, counteracting its inhibitory effect on RhoA/Rho kinase pathway. This may provide the basis for the possible use of this drug in the treatment of overactive bladder.

YIP1 ISOFORMS IN SKELETAL MUSCLE.

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The internal membrane system of skeletal muscle cells, including the endo/sarcoplasmic reticulum, the T-tubules and the Golgi apparatus, presents a very precise spatial organization with respect to the myofibrils. Within the endo/sarcoplasmic reticulum, the transitional endoplasmic reticulum (ER) is localized to the Z lines and at the perinuclear region, the "canonical ER" is mainly located in the terminal cisternae, whereas the Golgi appears in spots along the fibers and around the nuclei.
In the effort to identify genes involved in the development and/or maintenance of this highly regular membrane organization, we isolated a cDNA encoding a protein homologous the yeast Yip1p, named Yip1B. Yip1p is a protein important for the biogenesis of ER-derived COPII transport vesicles. The mammalian homolog of Yip1p includes two members, Yip1A and Yip1B. Yip1A is ubiquitously expressed and is enriched in the ER exits sites. Yip1B represents a muscle-specific isoform.
By RT-PCR, we found that striated muscle expresses both the Yip1 isoforms. To unveil the function of Yip1B with respect to the ubiquitously expressed Yip1A, we produced polyclonal antibodies against Yip1B. By Western blot, the antiserum was able to recognize the endogenous protein in striated muscle and differentiated C_2C_{12} cells, but not in undifferentiated C_2C_{12} and other tissues, as expected. Moreover, the protein resulted enriched in the skeletal muscle microsomal fraction, confirming its association to the membrane. Immunofluorescence experiments on skeletal muscle revealed that the Yip1B protein is localized both in the Z band and in spots along the fiber. The Z-band localization is in overlay with the Sec23 marker, whereas the spots are in overlay with the cis-Golgi marker GM130.

CALSEQUESTRIN-1 CONTROLS CA^{2+} RELEASE UNIT ARCHITECTURE AND MODULATES THE AMPLITUDE OF THE CALCIUM TRANSIENT INDUCED BY ELECTRICAL STIMULATION IN FAST-TWITCH MUSCLE FIBRES .

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Amplitude of calcium (Ca^{2+}) transients and structure of Ca^{2+} release units (CRUs) are significantly altered in Calsequestrin1 (CS1)- null mice (*Paolini et al 2007*). Structural alterations induced by lack of CS1 and differences in expression of CS2, Triadin, Junctin were detected in CS1-null FDB muscles in comparison to wild type (WT). To verify if these modifications are the direct result of CS1 ablation, exogenous CS1 was expressed in adult FDBs by *in vivo* electroporation. CS1 identification and correct targeting to CRUs was verified by confocal microscopy, whereas restoration of CRUs architecture - i.e. shape and width of junctional SR (jSR) containing CS1 - was assessed by electron microscopy. Exogenous CS1 was correctly targeted to CRUs and positioned at the jSR, in close proximity of Ca^{2+} release sites, the ryanodine receptors. At proteomic level CS2 and both the CS-binding proteins Triadin and Junctin did not change upon CS1 expression. Moreover, Ca^{2+} transients induced by electrical stimulation were recorded in mock-transfected , and CS1-transfected fibers: average peak height and baseline showed a significant increase upon CS1 expression and "rescued" transients resembled those of control WT fibers. In conclusion, the present results provide strong evidence that expression of CS1 directly controls size of jSR terminal cisternae, influence resting cytosolic Ca^{2+} and modulates the amplitude of Ca^{2+} transient in response to electrical stimulation in fast-twitch muscles**.**

HEAT- AND ANESTHETIC-INDUCED SUDDEN DEATH IN CALSEQUESTRIN-1 KNOCKOUT MICE.

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Malignant hyperthermia (MH) and exceptional/environmental heat stroke (EHS) present as life threatening crises triggered by volatile anesthetics and strenuous exercise and/or high temperature, respectively. MH and EHS episodes are characterized by uncontrolled elevations in core body temperature, rhabdomyolysis and are observed to a greater extent in males. Many, but not all, families (70-80%) diagnosed with MH susceptibility (MHS), and a few with EHS, are linked to mutations in the gene that encodes the Ca^{2+} release channel (RyR1) of muscle. Thus, other MH gene loci remain to be identified. In the present paper, we investigated whether a MH/EHS-like phenotype results from deficiency in skeletal muscle calsequestrin (CASQ1), a SR Ca^{2+}-binding protein that modulates RYR1 function. Strikingly, both halothane and heat challenge trigger lethal MH/EHS-like episodes characterized by elevated core temperature and rhabdomyolysis in male CASQ1-null mice. These episodes are prevented by prior dantrolene administration, the standard treatment for MH episodes in humans. Skeletal muscle from CASQ1-null mice exhibit increased contractile sensitivity to caffeine, temperature-dependent increases in resting Ca^{2+}, and an increase in the magnitude of depolarization-induced Ca^{2+} release. Together, these findings validate CASQ1 as a candidate gene for linkage analysis in MH and EHS families where mutations in RYR1 are excluded.

SESSION V

SKELETAL MUSCLE MECHANICS

STRUCTURAL CHANGES IN THE MYOSIN MOTORS DURING ACTIVATION AND FORCE GENERATION OF MUSCLE.

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Structural changes undergone by the myosin motors upon activation of muscle are investigated by X-ray interference during the transition from the resting state to the isometric contraction of single muscle fibres. Isolated intact fibres from frog skeletal muscle (2.1µm sarcomere length, 4°C) were mounted vertically at the beamline ID2 of the ESRF synchrotron (Grenoble, France) between a loudspeaker motor and a capacitance force transducer. 2D diffraction patterns were collected on a CCD detector with 5ms time resolution during the development of the isometric tetanus.
After the start of stimulation, the intensity of the M3 reflection, originating from the 14.5nm axial repeat of the myosin motors, first decreases to 30% (at 50 ms) of its resting value, then increases to a steady value 70% of that at rest. The fine structure of the M3 shows one major peak at rest and two peaks of comparable intensity at the tetanus plateau (Linari *et al.*, 2000, *PNAS*, 97:7226). The structural model of the thick filament that best fits the X-ray signals assumes that: (1) the motors diffract coherently (rest-active transition occurs on the same thick filament); (2) the number of active motors increases at the expenses of the number of rest-like motors in proportion to the isometric force (Brunello *et al.*, 2006, *J. Physiol.*, 577:971); (3) the conformation of active motors is independent of the level of force and strain on the thick filament.
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MECHANICAL PROPERTIES OF THE ACTOMYOSIN BOND DURING THE QUICK FORCE RECOVERY IN SINGLE FROG MUSCLE FIBRES.

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It is assumed that in muscle contraction, force generation at molecular level occurs in two steps: a relatively slow attachment of myosin heads to actin without force, followed by a fast process, the myosin "power stroke", which stretches the elastic component of the half-sarcomere and develops force. We characterized the mechanical properties of the actomyosin bond by applying to intact activated frog muscle fibres, fast stretches (~25 $nmhs^{-1}$ amplitude and ~350 µs duration) which forcibly detached the crossbridges. Stretches were applied before and after a conditioning stretch or release length change (~4 $nmhs^{-1}$ amplitude), during the quick force recovery which it though to represent the synchronized power stroke of the crossbridges. The rupture force of the crossbridge ensemble (critical force, P_c) and the sarcomere elongation at P_c (critical length, L_c) were measured. Experiments were performed at 5°C; force and sarcomere length were recorded with a fast force transducer (~40 kHz natural frequency) and a striation follower device. In contrast with the data obtained previously on the tetanus rise (Bagni et al., 2005), P_c was almost independent of the tension developed by the fibre at the time of the stretch and L_c was not constant but changed with a characteristic time course. In agreement with the theory we show that the crossbridge state at the end of the quick recovery is characterized by a lower potential energy respect to isometric state. The individual crossbridge rupture force does not change significantly. The forced rupture of the actomyosin bond produced by fast ramp stretches occurs through the reversal of the process leading to force generation.

THE MOLECULAR BASIS OF MUSCLE BRAKING.

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The braking action of active muscle consists in the ability to resist an increase in load up to twice the isometric force, by quite small increases in lengthening velocity (Katz, *J. Physiol. 96:45,1939*). During muscle stretch the half-sarcomere (hs) stiffness increases suggesting that force enhancement by stretch is related to the recruitment of an additional elastic structure (Linari et al., *J. Physiol. 526:589, 2000*). X-ray interference changes indicate that the stiffness increase is accounted for by a very rapid attachment to actin of the second motor domain of the myosin molecules with the first motor domain already attached in the isometric contraction (Brunello et al., *PNAS, 104: 20114, 2007*). The mechanism is further investigated here by using single frog fibre mechanics (*Rana esculenta*, 4 °C, 2.1 μm sarcomere length). Stretches between 2 and 8 nm hs^{-1}, complete within 100 μs, were applied at the tetanus plateau (T_0) and the fraction of new motors relative to the isometric number (*f*) was determined either at the peak of the force response to stretch (T_1) or at the end of the quick phase of force recovery, 2 ms after the stretch (T_2). We show that: 1) for stretches <5 nm, independently of the phase of the force response to stretch, *f* depends solely on the size of the axial distortion (Δz) of the attached motors; 2) at T_1 *f* saturates at 0.3 for stretches >5nm; 3) at T_2 *f* increases with Δz up to a maximum value of 1; 4) the dependence of *f* on Δz remains the same even after halving the isometric force and the number of actin attached motors with the addition of 0.5 μM BTS to the bathing solution. These results support the idea that the distortion of the attached motors promotes the attachment of the partner motors and indicate an upper limit ($\sim 10^4\ s^{-1}$) for the rate of the recruitment process. Supported by MiUR, NIH (USA).

MECHANICAL PROPERTIES OF SINGLE INTACT SKELETAL MUSCLE FIBRES ISOLATED FROM WILD-TYPE AND MLC/mIGF-1 MICE.

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In this comparative study we analyzed the most important mechanical properties of single intact muscle fibres of wild-type (WT) and MLC/mIgf-1 (TG) mice, in which the localized Igf-1 transgene expression sustains hypertrophy (Musarò et al., *Nat. Genet.* 2001). In additional to the classical mechanical parameters, we focussed on "static stiffness" (SS), a non crossbridge calcium-dependent stiffness previously identified in activated frog muscle fibres (Bagni et al., *J. Physiol.* 2002). Single fibres were dissected with the intact plasmatic membrane from flexor digitorum brevis muscle and mounted with microclips gripped on tendons in an experimental chamber (~23°C) between a force transducer and an electromagnetic motor to apply fast stretches. Sarcomere length was measured by means of a videocamera. The results showed that TG fibres exhibited an increase in diameter and absolute force, but specific force and force-velocity relation were not significantly different than WT fibres. The plateau of length-tension relation was in agreement with the length of the myofilaments reported in literature. SS was present either in WT or in TG fibres but its characteristics were slightly different. These data show that the only significant mechanical difference between WT and TG fibres is in the SS properties. This may be related to a different compliance or different calcium response of the structure responsible of the SS. In addition, these experiments show that single intact fibres could be successfully used to study the mechanical properties of WT and transgenic mouse muscle under various conditions such as aging or diseases.

TEMPERATURE DEPENDENCE OF MGATP AND MGADP SENSITIVITY OF SLIDING VELOCITY ON FAST AND SLOW RAT MYOSIN ISOFORMS.

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It has been suggested that the rate of ADP release from acto-myosin and the rate of acto-myosin dissociation by ATP may play different relative roles to define unloaded shortening velocity (Vo) of slow and fast myosins (Nytray et al. 2006) and their role might change with temperature. In this study we have used the *in vitro motility assay* to study the effect of MgATP and MgADP on the sliding velocity of actin (Vf) on slow and fast skeletal myosin isoforms from rat at 15, 20, 25 and 35°C. MgATP concentrations varied in the range 0.01mM and 2mM in the absence and in the presence of 2mM MgADP. The ATP sensitivity of Vf was higher in fast than in slow myosin at all temperatures and increased increasing temperature in both isoforms. The presence of MgADP decreased Vf of both isoforms at all temperatures. In slow isoform the inhibitory effect of ADP increased from 15 to 25°C and remained constant from 25 to 35°C. On the contrary, in fast isoforms the inhibitory ADP effect increased from 15 to 25°C and decreased from 25°C to 35°C. These results suggest a different role of the rate of acto-myosin dissociation by ATP and of the rate of ADP release in modulating Vo of slow and fast myosins.

SESSION VI

SKELETAL MUSCLE ADAPATATIONS AND EXERCISE I

IDENTIFICATION OF 2B, 2EO AND LARYNGEAL MYOSIN HEAVY CHAIN ISOFORMS IN HUMAN PRE-SOMITIC SKELETAL MUSCLES.

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In this work we compared "specialized" muscles as masticatory (*masseter*), extraocular (*rectus lateralis*) and laryngeal (vocalis portion of *tyroaritenoideus, interarytenoideus, cricothyroideus* and the posterior portion of *cricoarytenoideus*) with *vastus lateralis*, atrial myocardium and fetal psoas muscle (21 weeks) which are used as internal controls since their specific MyHC expression profile (1, 2A, 2X, □-cardiac and developmental isoforms, respectively). Combining isoform-specific MyHC TaqMan probes with SDS-PAGE electrophoresis we provided a reliable MyHC classification in all muscles studied, both in term of mRNA and protein expression. However, we encountered a high MyHC expression diversity depending on the sampling site within the muscle and individual variability. Our results indicate that specialized muscles express skeletal isoforms (1, 2A and 2X) together with MyHC peculiar isoforms: -masseter expresses □-cardiac and perinatal/neonatal; -laryngeal muscles express □-cardiac, laryngeal, perinatal and embryonic; -extraocular muscles express all MyHC isoforms except for the perinatal. Overall, this study shows that the flux of MyHC mRNA examined by Real Time PCR is very irregular compared to the corresponding protein confirming that it is essential to investigate both molecules to gain consistent results.
Interestingly our data show for the first time the expression, at protein level, of the 2B isoform in *rectus lateralis* muscle and confirm the presence of a novel laryngeal isoform.

DIFFERENT INTRINSIC PROPERTIES OF YOUNG AND AGED HUMAN SATELLITE CELLS.

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During aging, skeletal muscles undergo a decline in functional capacity due to loss of regenerative ability of satellite cells (SCs), the quiescent stem cells located beneath the basal lamina surrounding each myofiber. There is debate about the influence of age-related extrinsic factors on SC efficiency (the SC niche) *vs* age-related intrinsic cellular properties of the SCs (1).
In the present work we analyzed several parameters of SCs derived from biopsies from *Vastus Lateralis* muscle from both healthy non-trained young and aged humans [male and female, divided into young (mean age 31.6 ± 3.6 years; n=5) and aged (mean age 77.3 ± 5.8 years; n=7)].
Aged SCs showed impaired differentiation ability [i.e., lower extent of fusion into myotubes and reduced expression of myogenin and myosin heavy chain, when cultured in differentiation medium (DM)], compared with young SCs, and were characterized by the following: i) a stronger expression of S100B, a Ca^{2+}-binding protein the overexpression of which has been found to interfere with myoblast differentiation (Tubaro C et al., submitted for publication); ii) undetectable levels of full-length RAGE (receptor for advanced glycation end products) protein, a multiligand receptor the engagement of which enhances myoblast differentiation (2), in growth medium (GM), and cytosolic instead of membrane localization of RAGE in DM; and iii) lower expression levels of MyoD and Pax7 transcription factors, in both GM and DM.
These data point to an important role for intrinsic factors, besides extrinsic factors, in defective SC function during aging.

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AGE-DEPENDENT CHANGES OF SIGNALING PATHWAYS OF MUSCLES FROM α-SARCOGLYCAN-DEFICIENT MICE.

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The dystrophic muscle originated from defects of the dystrophin-glycoprotein complex is usually characterized by muscle hypertrophy and altered fiber-type profiles. In order to reveal the signaling pathways responsible for these events, we investigated a number of key elements involved in the control of muscle diversity and plasticity. The study was performed in EDL, soleus, and diaphragm isolated from control and *Sgca*-null mice, the □-sarcoglycan-deficient mouse model of type 2D limb-girdle muscular dystrophy, a progressive muscle wasting disorder. Changes of morphological, physiological and biochemical properties in adult (6 months) and old (18 months) mice were correlated with the expression and activity of different factors and enzymes. Muscles of adult *Sgca*-null mice show a significant larger mass compared to controls. The mean CSA of adult EDL fibers was 40% smaller than age-matched control, but returned within control values in old mice. The mean CSA of diaphragm fibers was about half that of controls, independently of age. Clear-cut changes of MyHC composition were evident between dystrophic and control muscles and during aging. The mean level of activated Akt and calcineurin, the key signal proteins controlling muscle mass and diversity, is higher in adult dystrophic diaphragm than in control. Cytochrome c, index of mitochondrial content, increases with age in both control and dystrophic muscles, while PGC-1□, regulator of oxidative metabolism, decreases with aging. This preliminary study shows that both aging and dystrophy perturb the expression of muscle signaling proteins.

FOXO3 AND FOXO1 CONTROLS DIFFERENT ATROPHY-RELATED GENES.

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FoxO1, 3 and 4 belong to the superfamily of Forkhead transcription factors and are expressed in skeletal muscles. FoxOs are involved in several cellular functions including activation of an atrophy program which leads to muscle loss. FoxO factors are under IGF-1/PI3K/AKT control and in physiological conditions are phosphorylated by AKT and sequestered into the cytoplasm. On the other hand, in many pathological conditions FoxOs are dephosphorylated, translocate into the nucleus and interact with promoters of target genes. Since it is known that FoxO1 and FoxO3 overexpression causes muscle atrophy we asked whether they control the same set of atrophy-related genes or whether every FoxO member regulates a peculiar subset of genes. To address this question we used Chromatin immunoprecipitation (ChIP) approach. First we determined which are the FoxO1 and FoxO3 binding site on promoter of Atrogin-1, the critical atrophy-related ubiquitin ligase. ChIP was performed on endogenous FoxO3 and 1 in atrophying adult muscles during fasting. We show that FoxO3 binds to specific regions of Artogin-1 promoter and induces histones acetylation. Surprisingly these sites are specific for FoxO3, since FoxO1 does not or weakly interacts. Using dual-luciferase-assay, we confirmed the preferential FoxO3-mediated transactivation of Atrogin-1 promoter. All these results suggest that FoxO members potentially control different subset of atrogenes. To identify these genes, we applied ChIP on Chip technique that extends the analyses of protein-DNA binding to promoter regions of whole genome. By different ChIP on Chip experiments we identified the FoxO3-dependent and FoxO1-dependent atrogenes. The overlap of these two groups shed a light into the mechanisms of FoxO-mediated muscle atrophy and define the critical genes that are required for the atrophy program.

VARIATIONS IN CROSS SECTIONAL AREA AND MYOSIN CONTENT IN SINGLE HUMAN MUSCLE FIBRES FOLLOWING FIVE WEEKS BED REST.

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Following bed rest (BR) and in general disuse both skeletal muscles in vivo and single muscle fibres in vitro can loose disproportionately more force than size, i.e. a decrease in specific force can occur. In elderly immobilized subjects one of the determinants of such phenomenon is a larger loss of myosin content than of CSA, i.e. a decrease in myosin concentration. The goal of this study was to assess whether disuse can determine a similar phenomenon in young healthy subjects. Biopsy samples were taken from the vastus lateralis muscle of 10 healthy young subjects pre-5wk BR and post-5wk BR and divided in small bundles which were stored at -20°C in skinning solution plus glycerol 50%. Individual fibres were dissected from muscle bundles and divided in two portions. The first (~0.2mm long) was used for fibre typing based on MHC isoform determination by SDS-PAGE; the second part (average length 2.8mm) was used for volume and myosin content determination using a refinement of a previously developed technique based on quantitative electrophoresis (D'Antona et al 2003). Biopsy samples of 4 subjects have been studied so far (20 fibres per subject pre-BR and post-BR). CSA was found to be significantly lower post-BR than pre-BR in both type 1 (3396±915 SEM n=17 post-BR vs 4902±1752 n=25 pre-BR; -31%) and type 2A (4396±1463 n=22 pre-BR vs 5616±1352 n=24 post-BR; -22%) fibres. In the same fibres, myosin concentration was found to be significantly lower post-BR (130±54 µM) than pre-BR (176±72.17 µM) in type 1 fibres only. No difference was observed among type 2A fibres. The analysis of a large percentage of the fibres which were hybrid and of the myosin actin ratio of all fibres are ongoing.

ANY ROLE FOR HOMER 2 IN SKELETAL MUSCLE ATROPHY AND PLASTICITY?

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Homers represent a family of scaffolding proteins of which are known three genes and many splicing isoforms. Several lines of evidence point to Homers as modulators of Ca^{2+}signalling in skeletal muscle. The EVH1 domain of Homers interacts with and regulates the activity of several proteins within Ca^{2+}-signaling complexes in an isoform specific manner. In skeletal muscle Homer 1 appears to interact with RYR1.
It is known that: **a)** In C2C12 myotubes, Homer 2b modulates IP_3R- and RyR-mediated Ca^{2+} release to enhance NFAT transcriptional responses thus enhancing transcription of slow-twitch muscle specific genes; **b)** Homer 2, but not Homer 1, physically interacts with NFAT in HEK cells and modulates NFAT nuclear translocation in T cells, and **c)** NFAT is known to be a regulator of muscle differentiation. Based on the foregoing, we studied the relationship between Homer 2 and NFAT in slow-twitch skeletal muscles, in order to assess whether Homer 2 plays a causative role in slow-twitch fiber type specialization and in the atrophy process evoked by either denervation or disuse.
We found that: a) Homer 2a/b is present only in the slow-twitch phenotype of skeletal muscle; b) Homer 2a/b aggregates with NFAT¸ and c) denervation atrophy and disuse atrophy of slow-twitch muscles are accompanied by a marked and early down-regulation of Homer 2a/b both at the protein and mRNA level. Based on present and recent data, it appears that transition in Homer composition plays a role in skeletal muscle adaptation.

COMBINED BIOPHYSICS, GENETIC AND PROTEOMIC ANALYSIS OF THE EFFECT OF STATIN AND FIBRATE TREATMENT ON RAT SKELETAL MUSCLE.

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Statin and fibrate can produce a variety of muscle-related complaints in treated patients. We previously demonstrated that lipophilic statins and fibrates affect skeletal muscle function by reducing resting chloride conductance (gCl) (Pierno et al, *Br J Pharmacol* 2006). The gCl sustained by the ClC-1 chloride channel controls the sarcolemma electrical stability and its reduction can produce myotonia-like symptoms. Here we analysed the time-dependent effects of fluvastatin (20 mg/kg) and fenofibrate (60 mg/kg) on Extensor Digitorum Longus (EDL) muscle gCl measured by two-microelectrode current clamp method. We observed a gCl decrease in a time-dependent manner, being significantly lower after 1 week (2464±66 □S/cm^2, n=46 and 2510±53 □S/cm^2, n=50 in fluvastatin and fenofibrate treated rats, with respect to 2706±83 □S/cm^2, n=27 of control). To address the causes of gCl reduction we also investigate the effect of statin and fenofibrate chronic treatment on ClC-1 gene expression by real-time quantitative PCR. The results showed a marked decrease in ClC-1 mRNA expression in both fluvastatin and fenofibrate treated animals which contributes to gCl reduction. We hypothesize that statins or fibrates can modify the expression of proteins which can be essential for muscle function. At this aim we analysed the proteomic map of EDL muscle from rat treated with fluvastatin (20mg/kg), atorvastatin (10mg/kg), fenofibrate (60mg/kg) and with combined fluvastatin (5mg/kg) plus fenofibrate (30mg/kg) by two-dimensional gel electrophoresis (Gelfi et al, *J Proteome Res* 2006). The comparison of the proteome maps showed 40, 74, 60 and 76 protein spots differently expressed in the above mentioned treated groups, with respect to control. The identification of each spot by mass spectrometry is in progress. The proteomic analysis will hopefully identify the protein targets of the myopathic process and help to clarify the mechanisms underling such major side effect of the therapy.

ACTIVATION OF AN ATROPHIC PROGRAM BY C-FLIP OVER-EXPRESSION IN MOUSE SKELETAL MUSCLE.

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c-Flip is a known anti-apoptotic molecule inhibiting Fas signaling. A transgenic (Tg) mouse over-expressing c-Flip (Tg-c-Flip) under trSra8 promoter has been previously constructed and a reduced heart-hypertrophy was reported. In the present study we demonstrate that trStra8 promoter is activated both *in vivo* and *in vitro* in undifferentiated skeletal muscle cells and that phosphorylation of both Akt and GSK3□ is strongly reduced the Tg-c-Flip mouse. We thus hypothesized an impairment of the skeletal muscle physiology in the Tg-c-Flip mouse. In fact, a marked decrease of both fibers cross sectional areas (43% inhibition) and muscle mass (20% inhibition), an evident mitochondrial proliferation as well as a two-fold increase of proteins oxidative modifications were shown in the Tg-c-Flip mouse *vs* wild type (WT). The GSK3□ activity target □-catenin was also decreased, correlating with apoptosis signs such as citochrome c release and caspase-3 activity located outside the myofibers, where satellite cells localize. The concomitant reduction of the satellite specific marker Pax-7 (30% inhibition in the adult mouse) led us to hypothesize that the marked skeletal muscle atrophy observed in the Tg-c-Flip mouse may be related to a reduction of the satellite cells pool. Altogether these data demonstrate a novel role for c-Flip in regulating skeletal muscle physiology and remodeling.

SESSION VII

SKELETAL MUSCLE ADAPATATIONS AND EXERCISE II

EVALUATION OF HRR IN RELATION TO DIFFERENT POSITION IN PROFESSIONAL SOCCER PLAYERS.

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The valuation of Heart Rate Recovery (HRR) after exercise represents a simple and minimal invasive system used for the valuation of vagal activity (**Imai** *1994*). The autonomic contribution in HRR after exercise is described in literature (**Harun** *2006)*. Many authors have noted better HRR values in well trained athletes compared to sedentary ones, they have also evidenced HRR correlation with exercise capacity (**Cole** *1999).* In Sport Medicine HRR was already considered as an important parameter for the valuation of cardiovascular fitness, but there are no informations on the correlation between HRR and professional sportsmen.
The aim of our study was the valuation of the autonomic cardiac function (vagal-sympathetic balance) in professional soccer players, with particular attention to the differences between keepers and other players.
In our study we have measured HRR after 1 and after 2 minutes of active recovery (HRR1 and HRR2) from exercise stress test, in 87 professional soccer players. HRR1 was significantly slower (22,0) in keepers in comparison with other roles (28,34) ($p<0.01$). Keepers had also an HFr (65.25) significantly higher than other players (57,84) ($p<0.05$). There weren't significant differences between other roles (forwards 25,92; midfielders 30,27; defenders 27,92).
These data show a better autonomic function in roles with an alternate aerobic-anaerobic activity compared to roles with prevalent anaerobic alactic metabolism. These results, novel in the matter of the examined athletes typology, agree with other data in literature supporting the positive action of aerobic-anaerobic physical activity on autonomic cardiovascular system adjustment.

ELECTRICAL AND MECHANICAL RESPONSE OF FINGER FLEXOR MUSCLES DURING VOLUNTARY ISOMETRIC CONTRACTIONS IN ELITE ROCK-CLIMBERS.

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To determine the differences between rock-climbers and controls in finger flexor motor units (MUs) features and activation strategy, eleven climbers and ten controls volunteered for the study. After maximal voluntary contraction (MVC) assessment, 5 levels of isometric contractions at 20%, 40%, 60%, 80% and 100% MVC were performed. During contractions, EMG and mechanomyogram (MMG) were recorded, from which the root mean square (RMS) and mean frequency (MF) were calculated. Climbers showed significantly higher MVC values. EMG rms was statistically higher in climbers than in controls from 60% to 100% MVC. In climbers, MMG rms increased up to 80% MVC, whereas in controls it increased only up to 60% MVC. MMG MF was higher in climbers than in controls at all exercise intensities ($p<0.05$). EMG-MMG combined analysis revealed significant differences in MU activation strategy between the two groups. The results are compatible with a shift of climbers' muscles toward faster MUs.

DETERMINATION OF LACTATE THRESHOLD DURING INCREMENTAL WORKLOAD IN SALIVA.

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Aim: the aim of the present study was to determine the salivary lactate threshold (*LT*) and to compare the anaerobic threshold (*AT*) values based on computerized calculations of different respiratory gas indices with those obtained from salivary lactate measurements in physically active subjects.
Methods: Fourteen healthy male subjects (age, 19.5 ± 3.9 years) performed a stepwise incremental cycle ergometer test until exhaustion. The Respiratory gases were measured continuously, and salivary samples were drawn every 2 min for subsequent determinations of lactate concentration.
Results: At rest, *HR* was 89.8 ± 6.8 bpm; VO_2, 6746 ± 184.7 mL x min^{-1}; the respiratory exchange ratio (*RER*), 0.8 ± 0.01 VCO_2/VO_2; lactate, 0.2 ± 0.1 mM. The *AT* was determined, based on *RER* > 1. Subjects reached the *AT* at the workload intensity of 162.1 ± 36.0 W. Within the *AT*, *HR* was 151.1 ± 11.1 bpm; VO_2, 2240.4 ± 494.8 mL x min^{-1}; lactate, 0.5 ± 0.2 mM. At exhaustion *HR* was 170.8±14.8 bpm; VO_2, 2928.0 ± 505.6 mL x min^{-1}; *RER*, 1.1 ± 0.01 VCO_2/VO_2; lactate, 1.3 ± 0.7 mM. As expected, the curve representing measurement data of salivary lactate run flat at the beginning until individuals reached the *AT*. Significant correlation between gas exchange indices and salivary lactate was found for VCO_2 at R5' ($r = 0.56$, $p < 0.05$), for *RER* at 230 W ($r = 0.49$, $p < 0.05$) and for V_E/VO_2 at 230 W ($r = 0.55$, $p < 0.05$).
Conclusion: salivary *LT* correlated well with ventilatory and gas exchange-based determination of *AT*. Therefore, salivary lactate-based *AT* determination represents a reliable, non-invasive and simple method able to correctly predict the onset of anaerobic metabolism during exercise.

POSTER

Muscle Disease and Therapies

CACHECTIC MUSCLES EXHIBIT BOTH AN INCREASED SUSCEPTIBILITY TO DAMAGE AND A REPARATIVE DEFICIT.

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Cachexia is a severe skeletal muscle wasting syndrome affecting most cancer and other chronic patients. We analyzed different muscles in colon carcinoma-C26 bearing mice and controls. We also compared mice hosted in standard versus wheel-equipped cages. We quantified muscle damage with multiple methods (histological evaluation of EBD+ fibers, fluorometric quantification of EBD uptake, CK serum levels) and found that the tumor burden induced a statistically significant increase of skeletal muscle damage. We noted that physical exercise increased the degree of fiber damage although to a different extent in different muscle. The extent of fiber damage linearly correlated with fiber necrosis (shown by immunohistochemistry for macrophage markers). This phenomenon resulted in a significant decrease in the total fiber number in C26-bearing, exercised mice. We investigated the regenerative capacity of cachectic muscles and found that, in spite of elevated Pax7 and MyoD expression in C26-bearing mice, increased damage is not accompanied by efficient regeneration (shown by fibers with centrally located nuclei). No modulation of later markers of muscle regeneration (such as desmin, myogenin and Embryonic MHC) occurs in C26 bearing mice. In addition, we found that the C26 burden altered the percentage of satellite cells in S phase suggesting a perturbation in their activation. In conclusion, we propose that uncoupling between increased muscle fiber necrosis and regeneration can lead to muscle wasting through loss of muscle fibers.

MAGIC FACTOR-1, A PARTIAL AGONIST OF MET, INDUCES MUSCLE HYPERTROPHY BY PROTECTING MYOGENIC PROGENITORS FROM APOPTOSIS.

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Abstract
Hepatocyte Growth Factor (HGF) is a pleiotropic cytokine that mediates a characteristic array of biological activities including cell proliferation, survival and morphogenesis. The unique receptor for HGF is Met, expressed by a wide range of tissues. Following muscle injury, HGF-Met stimulation plays a key role in promoting activation and early division of satellite cells. In culture, HGF stimulation promotes proliferation of muscle precursors thereby inhibiting their differentiation.
Magic-F1 (Met-Activating Genetically Improved Chimeric Factor-1) is an HGF-derived, engineered protein that contains two Met-binding domains repeated in tandem. It has a reduced affinity for Met and it elicits activation of the AKT but not the ERK signaling pathway. Magic-F1 protects myogenic precursors against apoptosis, increasing their fusion ability and enhancing muscular differentiation. Magic-F1 transgenic mice displayed constitutive muscular hypertrophy, improved running performance and muscle regeneration. Crossing of Magic-F1 transgenic mice with α-sarcoglycan knock-out mice or adenovirus-mediated Magic-F1 gene delivery resulted in amelioration of the dystrophic phenotype. Thus Magic-F1 represents a novel molecular tool to counteract muscle wasting in major muscular diseases such as cachexia or muscular dystrophy.

ROLE OF IL-6 IN SKELETAL MUSCLE ATROPHY.

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IL-6 cytokine is involved in several pathologies characterized by a progressive loss of muscle tissue, such as aging, cachexia and muscular dystrophy, revealing an important role of this cytokine in skeletal muscle homeostasis.
Recent studies, demostrated that IL-6 local infusion in tibialis anterior muscle of healthy rodent, results in a decrease of muscle total and myofibrillar fraction protein content, contributing to the development of strongly atrophic phenotype. *(Haddad F et al., 2005)*
Moreover, the NSE/h-IL6(+/+) transgenic mice show growth defects associated with high circulating levels of IL-6 expressed since birth. *(De Benedetti F et al., 1997)*
Terminally, IL6 deficiency abrogated satellite cells proliferation and myonuclear accretion in the preexisting myofibers by impairing STAT3 activation and expression of its target gene D1. *(Serrano AL et al; 2008)*
In our laboratory, preliminary results showed that 10 days old NSE/h-IL6 tibialis anterior and quardriceps muscles present a strong atrophic phenotype compared to Wt muscle.
In addiction, we performed molecular and histomorphometric analysis on trangenic NSE/h-IL6 mice of 1.5, 3.5 and 6 months old, to clarify the role of this cytokine in muscular atrophy during postnatal growth.
In addition we observed in NSE/h-IL6 transgenic mice at 3.5 and 6 months of age a maintenance of atrophic phenotype characterized by a decrease of total cross sectional area, by a reduction of myofibers size and by a minor total number of fibers compared to Wt muscles. These results demonstrate an important role of IL-6 in skeletal muscle atrophy and suggest an involvement of this cytokine in the modulation of postnatal muscle growth.

Cell Biology and Myogenesis

PKC THETA EXPRESSION/ACTIVITY IS REQUIRED FOR MUSCLE MAINTENANCE AND REMODELLING.

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Skeletal muscle maintenance is allowed by the balance between different intracellular signalling pathways, regulating muscle cells growth and differentiation.
Protein kinase C θ (PKC θ) is a member of the "novel" calcium-independent serine/threonin kinases PKCs (nPKC subfamily) and it is the PKC isoform predominantly expressed in skeletal muscle.
We have previously shown that inhibition of PKCθ activity specifically in muscle (mPKCθ K/R mice) results in insulin-resistance associated to obesity, due to the impairment of insulin signal transduction (Serra et al. *J. Cell. Physiol.* 2003); moreover, PKCθco-operates with calcineurin in the activation of slow muscle genes in culture (D'Andrea et al. *J. Cell. Physiol.* 2006).
We now observe that ablation of PKCθ expression (PKCθ KO mice) or activity (mPKCθK/R mice) leads to a significant reduction of muscle mass. Indeed, PKCθ expression is reduced in various models of muscle wasting. Moreover, when PKCθ mutants were crossed with a transgenic model of muscle hypertrophy (MLC/mIGF-1 mice) limb muscle mass and cross sectional area of muscle fibres were clearly reduced in mIGF1/PKCθ null mice, compared with mIGF1 littermates, suggesting that PKCθ expression is required for IGF-1-induced muscle hypertrophy.
We are currently employing different *in vivo* (i.e. muscle regeneration, mechanical overload) and *in vitro* (i.e. starvation and refed) models to dissect the PKCθ-dependent signalling pathways responsible for growth, maintenance and adaptation of skeletal muscle.

MESOANGIOBLAST REPROGRAMMING: PLURIPOTENCY VS MULTIPOTENCY IN MUSCULAR DIFFERENTIATION.

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Mesoangioblasts (Mabs) are a novel class of adult stem cells originally isolated from the mouse embryo dorsal aorta[1], that have been isolated also from adult skeletal muscle in mouse, dog and human. Stem cell therapy with donor mesoangioblasts has produced dramatic amelioration in dystrophic mice and dogs. However, the finite life span and replication capacity, limit the potential of genetic modifications and relative potency in muscle differentiation and homing. Recently, Induced Pluripotent Stem Cells (iPS) have been generated by the ectopic expression of a combination of transcription factors (Oct3/4, Sox2, c-Myc, and Klf4 or Oct3/4, Sox2, Nanog and Lin28), in both mouse and human fibroblasts[2]. The iPSs reproduce unique features of embryonic stem cells. Our attempt is to reprogram Mabs to overcome their limitations and obtain a large amount of progenitor cells and evaluate their ability in muscular differentiation. These cells could offer tremendous promise for developmental, pharmacological, disease specific studies, and not last for regenerative medicine. Anyway, major challenges must be faced before medical applications can be considered. Besides the specific competences still we need to refine the knowledge concerning stem cell biology. Improving migration, differentiation and immune modulation ability can really make the difference to achieve efficaciousness in adult stem cell therapies.
1 Cossu & Sampaolesi Trends Mol Med. 2007 13(12): 520-6
2 Yamanaka S. Philos Trans R Soc Lond B Biol Sci. 2008 363(1500): 2079-87

From the electrical impulse to the SR Ca^{+2} release

PHYSIOLOGICAL ROLE OF NG2 IN SKELETAL MUSCLES: *IN VIVO* AND *IN VITRO* STUDIES.

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The NG2 chondroitin sulfate proteoglycan is a membrane-associated molecule of approximately 500 kDa with a core glycoprotein of 300 kDa. It is expressed primarily by glial, muscle, and cartilage progenitor cells. It is assumed to play the role of connecting the interior and exterior environments of the cells. On the inside, NG2 may interact with cytoplasmic or cytoskeletal proteins while, on the outside, it may interact with the extracellular matrix. In particular, there is evidence that NG2 binds to type VI collagen via a protein-protein interaction. The interaction of NG2 with extracellular and intracellular ligands also regulates signaling events that are important for both cell proliferation and cell migration.
In this study, functional parameters of skeletal muscle contraction were determined in NG2 null mice *in vivo* and *in vitro*.
Force development was measured *in viv*o (grip test) and *in vitro* (intact isolated muscles) and resistance to fatigue was examined *in vivo* (treadmill) and *in vitro* (intact isolated muscles). In addition the excitation-contraction coupling, was studied by recording Ca^{2+} transient in single fibres of FDB.
The results obtained showed that in NG2 null mice the ability to develop force was preserved while resistance to fatigue was clearly reduced.

Muscle Mechanics

THE EFFECT OF PHOSPHATE ON THE MECHANICS AND ENERGETICS OF THE ISOMETRIC CONTRACTION OF SKELETAL MUSCLE.

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We study the relation between chemical and mechanical steps of the myosin-actin ATPase cycle in muscle by applying fast, sarcomere-level mechanics to skinned fibres of rabbit psoas muscle. We have demonstrated that the increase in [Pi] decreases the isometric force in the half-sarcomere by decreasing the number of actin attached myosin motors, without change in the average force exerted by each motor (Caremani et al., *Biophys. J*. in press).
A 'conventional' five-step scheme of the myosin-actin ATPase reaction (Dantzig et al., *J. Physiol.* 451:247-278, 1992), that implies myosin detachment from actin upon release of hydrolysis products and binding of a new ATP, is able to fit the results, but is contradicted by the finding that the isometric ATPase rate is decreased by Pi (Potma et al., *Biophys. J.* 69:2580-2589, 1995; Potma and Stienen, *J. Physiol.* 496:1-12, 1996) much less than in proportion to the reduction of the number of actin attached motors. This feature of the chemo-mechanical coupling can be fitted with a reaction scheme that provides that the myosin motor under the isometric load can detach at an early stage of the ATPase cycle, with Pi still bound to its catalytic site, and then release the hydrolysis products and bind another ATP. In this way the model predicts that in fast skeletal muscle the ATPase rate per myosin motor and thus the energetic cost of the isometric contraction increases with [Pi]. Supported by NIH (R01 AR049033), MiUR and ITB-CNR

EFFECTS OF CONSTANT LOAD ON THE WORKING STROKE OF SKELETAL MYOSIN.

M. Maffei, M. Capitanio, M. Canepari, D. Beneventi, F.S. Pavone, and R. Bottinelli.

Myosin II drives muscle contraction through interactions with an actin filament. In each cycle an ATP molecule is split and a filament displacement (or working stroke,WS) is generated. The WS produced by a single myosin head has been previously measured in isolated myosin molecules, but the effects of the high loads acting on the myosin molecule during muscle contraction could not be investigated. In fact, current single molecule techniques apply force with a delay of few milliseconds after actin-myosin binding, when the WS of skeletal muscle myosin has already been completed. We developed a new single molecule technique in which a constant force is continuously applied to the actin filament, so the delay between myosin binding and force application is abolished. This method resolves the development of the myosin WS under different loads with a high time resolution and detecting events as short as 100 μs due to a very high signal-to-noise ratio. We found that under loads in the range 1 to 10pN myosin can follow two distinct pathways in its interaction with actin: a population of very fast events (100 μs – 1 ms) in which myosin detaches from actin before producing any movement (duration does not depend on ATP concentration), and a second population of events where myosin steps and remains bound to actin for a time longer at lower ATP concentration. The amplitude of the myosin WS is smaller at increasing loads and vanishes at the isometric force (~5pN). The reduction in the mean WS amplitude is due to an increase in the population of events that do not produce movement, rather than to a reduction in the amplitude of the events that produce movement. Otherwise the rise time of the WS becomes longer as the force increases (~200 μs at 1.5pN, ~600 μs at 4pN). Above the isometric force, only the fast events that do not step remain.

IN VITRO STUDY OF MECHANICAL AND KINETIC PROPERTIES OF MYOSIN II FROM FROG SKELETAL MUSCLE.

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We provide for the first time the protocol for efficient extraction and conservation (one week) of myosin II from frog skeletal muscle, a methodological achievement that makes it possible to apply single molecule techniques to frog myosin II. With the *in vitro* motility assay, we estimate the sliding velocity of actin on frog myosin II and its modulation by temperature and substrate concentration. The *in vitro* parameters are integrated with the *in situ* mechanical and kinetic parameters of frog muscle myosin working as an ensemble in the half-sarcomere. By comparing the sliding velocity of the actin filament on a bed of frog myosins (V_F) with the shortening velocity determined in intact frog muscle fibres under different loads and their dependence on temperature, we identify the mechanical conditions that explains the differences between *in vitro* and *in situ* measurements. With the integrated approach we can define fundamental kinetic steps of the acto-myosin ATPase cycle *in situ* and their relation with mechanical steps. In particular we clarify the relation between the rate of ADP release and the rate of detachment of myosin from actin and their temperature dependence. Supported by MiUR, NIH (USA).

THE INTENSITY OF THE 2.73NM REFLECTION USED TO DETERMINE THE NUMBER OF ATTACHED MOTORS IN THE ISOMETRICALLY CONTRACTING MUSCLE.

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The number of myosin motors generating the isometric force is a fundamental constraint for the definition of the kinetics and mechanics of muscle contraction. Mechanical measurements on single muscle fibres indicate that 30% of the available myosin motors are attached in the isometrically contracting muscle (Piazzesi et *al*. 2007, *Cell* 131:784). This number relies on the estimate of the compliance of the myofilaments.

We have used a structural approach to obtain an independent estimate of the number of attached motors. Whole sartorious muscles from the frog *Rana pipiens* were mounted at rest length in a chamber containing Ringer's solution at 10°C at the BioCAT beamline (Advanced Photon Source, Argonne, IL-U.S.A.). The muscles were activated through electrical stimulation and the force was recorded with a muscle lever system type 300B (Aurora Scientific). X-ray patterns were collected out to 0.5 nm^{-1} in the reciprocal space, using 1s total exposures under both resting and isometrically contracting conditions.

The meridional reflection originating from the 2.73 nm repeat of the actin monomers along the thin filament is expected to increase in intensity when myosin motors attach to actin in order to generate force. We observed that during the isometric contraction the intensity of the reflection increases by a factor 2.1±0.2 relative to rest. This intensity change is reproduced by a structural model based on crystallographic-EM coordinates for the acto-myosin complex (Holmes et *al*. 2003, *Nature* 425:423) if the fraction of myosin motors attached to the actin filament during the isometric contraction is 0.4, a value somewhat higher than what indicated by mechanical measurements.

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Skeletal Muscle Adaptations and exercise

UNILATERAL HINDLIMB IMMOBILIZATION: A SIMPLE MODEL OF PROTEASOME-DEPENDENT ATROPHY IN MICE.

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We describe a new simple protocol of immobilization to induce muscle atrophy in lower hindlimb muscles in mice. Hindlimbs of C57BL6 mice were unilaterally immobilized for 7 days. Morphological analysis revealed that immobilization resulted in a significant reduction in muscle fiber size in all the muscles analyzed, soleus, anterior tibialis and EDL. By contrary, no significant changes in muscle fiber type content was detected, as measured by NADH-TR staining. At the molecular level, the expression of the muscle-specific ubiquitin ligases MAFbx/Atrogin-1 and MuRF1 genes was induced upon immobilization in all the muscles examined. Atrogines up-regulation was paralleled by down regulation of PGC-1α expression. PGC-1α is a well-know negative modulator of Atrogenes, as being protective to atrophy processes. In addition, immobilization induced the expression of the autophagy marker Bnip3. The observed characteristics makes it a reliable model for muscle atrophy; its availability in mice may be instrumental for future studies on therapeutical interventions of muscle atrophy in transgenic and mutant mice strains. We are currently applying this model to the PKCθ knock out mice, as compared to WT mice, to investigate the possible role of this kinase in the intracellular signalling regulating skeletal muscle response to various atrophy stimuli: immobilization, denervation and fasting.

TEMPERATURE EFFECTS ON CONTRACTILE PARAMETERS OF FELINE MUSCLE FIBRES.

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Contractile properties of mammalian skeletal muscle fibres are mainly determined by myosin isoforms and among myosin subunits by the the isoforms of MyHC (Myosin Heavy Chain). Several MyHC (Myosin Heavy Chain) isoforms are expressed in skeletal muscle fibres: a) fast or type 2 isoforms (2A, 2X, 2B); b) cardiac isoforms (beta/slow MyHC and alpha MyHC; c) specialized isoforms expressed in restricted muscle groups such as Extraocular and Masticatory or M MyHC gene. Masticatory or M-MyHC is a isoform only expressed in muscles derived from the first branchial arch, in the first place, jaw closer muscles, with a clear inter-species variability. Only sparse information is available on the contractile properties of M fibres, i.e. muscle fibres expressing M-MyHC.

In this study we compared the contractile properties of the fibres expressing common skeletal muscle myosin isoforms, slow and fast (2A, 2X) and the specialized fibres expressing M-MyHC in a species of domestic carnivore: the cat. This species is of great interest for biology as well as for veterinary medicine. The cat fibres, mainly glycolytic and fatigable, reflect its tipical locomotion and behaviour.

In this study, in each muscle fibre isometric tension (Po), unloaded shortening velocity (Vo) and rate of tension development (Ktr) were determined during maximal calcium activation. To evaluate temperature effect, Po and Ktr were determined at two different temperatures (12°C and 24°C). Fibres were then classified on the basis of the MyHC isoforms expressed, separated in gel electrophoresis.

The results obtained showed that Vo and Ktr increased regularly from slow to 2A to 2X fibres and that M fibres had Vo and Ktr values similar to 2A fibres. M fibres showed Po values significantly greater than any other fibre type. Temperature-sensitivity was similar in all fibre types. Densitometric analysis showed significative differences in myosin content between M fibres and other fibre types .

THE SMALL HSP αBCRYSTALLIN PLAYS A KEY ROLE IN THE RESISTANCE TO OXIDATIVE STRESS DETERMINED BY VEGF IN SKELETAL MYOBLASTS.

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Recent studies suggest that Vascular Endothelial Growth Factor (VEGF) and its receptors could have protective potential, as for example in preventing neuronal cell death from ischemia and promoting neurogenesis in vitro and in vivo. Indeed, it is well known that in different cellular types, as vascular smooth cells, keratinocytes, rat heart endothelial cells, VEGF expression is triggered upon the influence to different injuring factors, e.g., hypoxia, UV light, reactive oxygen species (ROS) or mechanical injury.
Regarding this hypothesis, we have already seen that myogenic cell line, stably transfected with hVEGF165 cDNA ($C2C12^{VEGF}$), has an enhanced cell survival and resistance to apoptosis after exposure to cytotoxic concentrations of H_2O_2 (100-700 μM). Moreover, we identified αB-Crystallin (αBCry), a member of the small Hsp family, as possible mediator of the anti-apoptotic effect exerted by VEGF.
In this work, we additionally investigated about the role of αBCry in the resistance to apoptosis induced by H_2O_2 in the myogenic C2C12 cells exposed to VEGF, and the molecular pathways involved in this process. To this aim, we first analysed the cellular and molecular response towards H_2O_2-induced apoptosis in C2C12 myoblasts exposed to different concentrations of exogenous VEGF (0-10ng/ml), and then we verified if the specific silencing of αBCry by RNA interference (RNAi) could modify the apoptotic response of the $C2C12^{VEGF}$ cell line.
The supplementation of culture medium with VEGF (1-10 ng/ml) determined a decrease of susceptibility to apoptosis induced in C2C12 myoblasts by cytotoxic concentration of H_2O_2 (up to 50% reduction in respect to control, $p<0.01$). This reduction was paralleled by an increase in the expression of the αBCry protein, but not of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, which levels were not significantly modified. In addition, after specific αBCry silencing in the $C2C12^{VEGF}$ cell line, the anti-apoptotic effect of VEGF disappeared, with a rescue of apoptosis susceptibility back to control levels.
Together, our data provide that VEGF, beside its angiogenic properties, is also involved in protecting muscle cells against apoptotic stimuli caused by free radicals. Moreover, we demonstrate that αBCry plays a key role in the molecular mechanism underlying the anti-apoptotic effect of VEGF in myoblasts.

METABOLIC AND PRO-APOPTOTIC EFFECTS OF SALMETEROL TREATMENT ON C2C12 AND L6C5 SKELETAL MUSCLE CELLS.

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Salmeterol is a long-acting β-2 adrenergic receptor agonist, producing smooth airway muscle relaxation and bronchodilation (1), that is usually prescribed for the treatment of asthma and chronic obstructive pulmonary disease.
β-adrenergic receptor agonists were demonstrated to induce muscle hypertrophy with still unknown mechanisms (2), suggesting a possible substance's abuse by the athletes. To prevent the doping effect, WADA decided to forbid the free use of Salmeterol in sport activity, still allowing its and others analogue molecules utilization under medical exemption. Salmeterol's role in improving muscle mass and performance and in the asthma-related death is still an open question (3).
The aim of this work was to investigate the possible metabolic and citotoxic effect of Salmeterol on L6C5 and C2C12 myoblasts.
Proliferant L6C5 and C2C12 cells were exposed to different Salmeterol concentrations (0,1 -10μM) for 6 - 72 hours, and analysed for cell growth and survival, metabolic enzymatic activities and apoptosis pathway.
2,5μM, Salmetorol induces an high statistical increase ($p < 0,01$) of MTS signal which is already detectable after 6hrs of exposure, maintained up to 24hrs, and then dramatically reduced after 48hrs.
At maximal MTS effect, Salmeterol induces an increase of glyceraldehydephosphate dehydrogenase, citrate synthase, 3-OH acylCoA dehydrogenase activities, and a decrease of lactate dehydrogenase activity, with significatively higher response in L6C5 myoblasts ($p< 0,01$). Interestingly, the anabolic effect of Salmeterol, not paralleled by increase of cell growth (MI), was neither related to increase in cell resistance, since the expression of the Bcl-xl anti-apoptotic protein resulted unaffected.
Nuclear fragmentation, TUNEL assay and the Trypan exclusion test, showed the statistical increase ($p< 0,01$) of apoptosis in both L6C5 and C2C12 myoblasts exposed for 48 and 72hrs at the highest Salmeterol concentration.
This pro-apoptotic effect was confirmed trough the presence of cleaved PARP and the lack of its native form on C2C12 differentiated myotubes.
In conclusion these results indicated a possible positive effect at low Salmeterol exposure towards increasing oxidative metabolic pathways and showing a clear cytotoxic and pro-apoptotic effect at high Salmeterol exposure on both proliferating and differentiated skeletal muscle cells.

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PROTEOME ANALYSIS OF PROTEIN EXPRESSION IN SKELETAL MUSCLES OF HINDLIMB UNLOADED MICE.

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We used the model of 14 days Hindlimb Unloading (HU) in mice to study the mechanisms underlying disuse induced skeletal muscle atrophy by proteomic analysis.
We analyzed soleus and gastrocnemius as an example of a slow and a fast hindlimb muscle respectively. In soleus, a marked slow to fast shift of MHCs isoforms and a significant decrease of CSA of both slow and fast fibres was found following HU. The proteome map of soleus of control and HU mice was defined and a differential proteome analysis was performed. More than 800 protein spots on each gel were detected by fluorescent staining. Only a little percentage of detected spots were differentially expressed in HU mice in comparison with control animals. Most of the differentially expressed spots were implicated in oxidative stress (Hsp, SOD1, PRDX6, CAH III). Data showed an up-regulation of SOD1 and a down-regulation of other defence systems: Hsp, PRDX6, CAH III, suggesting the presence of oxidative stress in hindlimb suspended mice and a damage of defence system against oxidative stress.

Gastrocnemius muscle showed a lower degree of HU induced atrophy and few changes in protein pattern. SOD1, PRDX6, CAH III were up-regulated , whereas no changes were found in HSPs. Since data target the oxidative stress as one of the main mechanisms underlining disuse induced atrophy, the HU14 mice were treated with an antioxidant (30 mg/kg/day Trolox) to counteract the effects of oxidative damage and differential proteome maps of soleus and gastrocnemius muscles were obtained.

FUNCTIONAL AND PROTEOMIC ANALYSIS OF HUMAN SKELETAL MUSCLE FOLLOWING NEUROMUSCULAR ELECTRICAL STIMULATION.

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Transcutaneous neuromuscular electrical stimulation (NMES) exerts significant effects on skeletal muscle phenotype and function through increase in muscle mass, force and exercise capacity. NMES has been used as a tool for muscle strength training in athletes, in addition to a conventional conditioning routine, and in rehabilitation programs. NMES reduces the risk of traumatic lesions and falls in subjects with high degree of sarcopenia and standardizes training. The impact of NMES on muscle mass and function has been described in detail, but no information are available on the molecular mechanisms underlying such adaptations. In this study, ten, young (18-35 years), healthy, male subjects were subjected to 24, 18-min sessions of isometric (bilateral) NMES of the quadriceps muscle over a period of 8 weeks with 3 sessions per week. Needles biopsies were taken from the vastus lateralis muscles pre- and post-training. Maximum voluntary contraction (MVC) and neural activation were found to be significantly higher post-training (+20% and +9% respectively). MHC isoform distribution showed a significant shift MHC-2X → MHC-2A → MHC-1. Real-time PCR analysis of changes in MHC expression showed the same pattern. □-cardiac, embrional and perinatal MyHC isoforms, considered as transitional isoforms, were expressed only at the levels of mRNA. Fluorescently stained proteomic maps showing ~600 spots were obtained pre- and post-training and differentially expressed proteins were identified and subdivided in different categories.