

amount of connective tissue in diaphragm muscles but the mean numbers of LF granules/mm³ of diaphragm muscles of *mdx* mice treated orally with EGCG and subcutaneously with the higher dose of EGCG were reduced by 27 ± 6% and 22 ± 5% respectively. However, the lower injection dose had no effect. Both routes of EGCG administration did not alter the amount of LF in EDL muscles. Integrated locomotor activities of *mdx* mice only increased significantly (by 55 ± 18%) with the higher s.c. EGCG dose. These results suggest that decreased LF formation in involuntary diaphragm muscle is a more reliable marker for monitoring the effectiveness of EGCG treatment than that in voluntary EDL muscle. Our previous and present results indicate that EGCG is a potent drug for limiting the degeneration of dystrophin-deficient muscles.

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P3.42

Contribution of reactive oxygen species generated through NADPH oxidase to abnormal calcium signals in dystrophic skeletal muscle cells: involvement of phosphatidylinositol-3-kinase and protein kinase C

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Although the molecular mechanisms leading to muscle degeneration in Duchenne muscular dystrophy are not fully understood, numerous studies point to a deregulation of Ca²⁺ homeostasis and an increase in the generation of reactive oxygen species (ROS) in the pathogenesis of the disease. We show here that primary cultures of dystrophic myotubes generated more ROS than normal ones upon stimulation by KCl or phorbol 12-myristate 13-acetate. This increase was associated with a greater NADPH oxidase (NOX) activity in dystrophic myotubes. Under these conditions, NOX activation was mediated by both phosphatidylinositol-3-kinase/phospholipase C and protein kinase C pathways. We confirmed the presence of mRNA transcripts for NOX1, NOX2 and NOX4 isoforms in cultured myotubes. Dystrophic myotubes expressed greater mRNA levels for NOX2, as well as for p22^{phox}, the membrane subunit associated with NOX1, NOX2 and NOX4, and for p40^{phox}, p47^{phox}, p67^{phox}, the cytoplasmic subunits associated with NOX2, as determined by real-time RT-PCR. NOX4 mRNA was not changed. Moreover, ROS generated by NOX contributed to the increased Ca²⁺ transients in dystrophic myotubes as the NOX inhibitors diphenylene iodonium and apocynin restored these transients to those of normal myotubes. This effect was associated with an increase of both plasma membrane Ca²⁺ entry and Ca²⁺ release from the sarcoplasmic reticulum. In conclusion, our findings support the idea that ROS elicited by NOX contribute to the abnormal Ca²⁺ signals in dystrophic myotubes.

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P3.43

Long term administration of green tea polyphenols and pentoxifylline to *mdx*^{5Cv} dystrophic mice: differential effects on muscle function and kyphosis

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DMD is a fatal muscle disorder caused by the absence of dystrophin and characterized by progressive muscle wasting. Weakness of the back muscles often causes postural alterations, impairing patients' respiratory function. Oxidative stress likely contributes to the pathogenesis. We have previously shown that antioxidants such as green tea polyphenols (GTP) and EGCG (the major GTP component) improved muscle structure and function of the *mdx*^{5Cv} mouse, a model for DMD. Pentoxifylline (PTX), a non-selective phosphodiesterase inhibitor and TNF α release inhibitor was used as a positive control and also proved efficacious. EGCG is about to be clinically evaluated on DMD patients. In this context, we investigated the effects on dystrophic mice of long-term oral administration (for 15 months, from 3-weeks to about 16-months of age) of GTP, EGCG, and PTX. As assessed at the end of the treatment period with a wire test (involving fore- and hind-limb force), the motor function was improved with the following potency: PTX > GTP > EGCG. Using micro-computed tomography, we determined that kyphosis, the characteristic spine deformity occurring in old dystrophic mice as a result of back and thorax muscle weakness, was prevented by GTP, to a lesser extent by EGCG, but not by PTX. Tomography also allowed us to identify calcifications of the Achilles tendons, which, to the best of our knowledge, have never been reported so far. These calcifications were about twice larger in dystrophic mice than in normal mice and were diminished with the following potency: GTP > EGCG ~ PTX. Similarly, GTP and EGCG were more potent than PTX at reducing creatine kinase in the plasma (an index of muscle membrane fragility) and at preventing the loss of force induced by repetitive tetanic contractions. Our results suggest that GTP/EGCG and PTX exert different effects on different muscle groups. A combination of EGCG and PTX may provide more benefit than each compound alone.

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P3.44

Green tea polyphenols enhance the motor performance of the *mdx*^{5Cv} dystrophic mouse and normalize calcium influx in muscle fibres

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Duchenne muscular dystrophy (DMD) is a fatal muscle disorder caused by the absence of dystrophin and characterized by progressive muscle wasting. Oxidative stress and excessive calcium influx are thought to contribute to the pathogenesis. Indeed, we have previously shown that dietary interventions with powerful antioxidants such as green tea polyphenols (GTP) and EGCG (the major GTP component) improved muscle structure and function of the *mdx*^{5Cv} mouse, a model for DMD. As clinical trials are being conducted with EGCG on DMD patients, we report additional therapeutic effects of GTP and EGCG on the dystrophic mouse. As in our previous study, 3-weeks old mice were given for 5–8 weeks a chow enriched with GTP, EGCG or pentoxifylline (PTX), a non-selective phosphodiesterase inhibitor and TNF α release inhibitor used as a positive control. GTP, EGCG, and PTX ameliorated spontaneous locomotor activity and performance in a wheel running assay, and decreased plasma creatine kinase activity (a marker of muscle membrane fragility). The manganese quench technique was used to measure the influx of calcium into muscle fibres isolated from FDB muscles and loaded with the calcium probe Fura-2. Pre-treatment of the mice with GTP, EGCG, or PTX reduced by up to two-thirds the excessive calcium influx in dystrophic muscle fibres in resting conditions. In contrast, acute exposure to GTP, EGCG, or PTX of fibres

prepared from untreated mice did not alter calcium influx, neither at rest, nor after KCl depolarization. Similar findings were obtained from diaphragm strips. The expression levels of candidate calcium channels and calcium-binding proteins are being determined at the protein and mRNA level. Our findings suggest that GTP, EGCG, and PTX act through genome-dependent mechanisms to decrease the expression and/or the activity of calcium channels overactive in dystrophic cells. This effect likely contributes to the overall improvement of motor function on the awake animal.

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P3.45

Modulation of SERCA in dystrophic mice muscle – role of oxidative stress

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Oxidative stress contributes to the pathogenesis of chronic muscular diseases including Duchenne muscular dystrophy (DMD). DMD is an X-linked muscular disease caused by the absence of dystrophin and characterized by muscle membrane fragility and abnormally elevated levels of intracellular calcium. Intracellular calcium levels play a role in signaling function and are modulated by calcium regulating proteins, including the Ca^{2+} -ATPase from sarco/endoplasmic reticulum (SERCA). Therefore we have investigated which structural and functional alterations of SERCA are involved in the abnormal Ca^{2+} homeostasis of skeletal muscle in mice with muscular dystrophy (mdx^{5CV}). Alterations of SERCA were analyzed in SR vesicles isolated from skeletal muscles of hind paws from both normal and mdx^{5CV} mice of ages 2 and 8 month (young and adult mice) respectively. ATPase activity of SERCA from mdx^{5CV} muscle was increased 1.3-fold in young mice and 1.6-fold in adult compared with control animals. Concerning kinetic parameters, maximum reaction velocity increase was observed in mdx^{5CV} mice indicating an increase in number of active enzyme molecules and structural modification of protein domains of SERCA in mdx^{5CV} muscle. Also the Ca^{2+} and ATP affinities of the enzyme were altered. Expression of SERCA1 and 2 isoforms in mdx^{5CV} muscle were significantly increased. We identified only small increase of the protein carbonyl formation in adult mdx^{5CV} mice. We have found less sensitivity to oxidation *in vitro* induced by HOCl but higher sensitivity to oxidation by ONOO⁻ and increase of nitration in mdx^{5CV} muscle. Taken together this suggests that the increase of SERCA activity in mdx^{5CV} mice may be induced by oxidative stress leading to resistance against ROS, increased expression of SERCA and changes of affinity of SERCA for substrates.

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P3.46

Doxorubicin affects calcium handling in dystrophic skeletal muscle cells

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Duchenne muscular dystrophy (DMD) is a progressive disease affecting 1/3500 male births and characterized by the absence of dystrophin due to a defect in the p21 band of the X chromosome (Monaco et al., 1986). Lack of dystrophin expression causes muscle

degeneration by a mechanism that remains elusive but has long been attributed to membrane defects and/or increased permeability to Ca^{2+} . A number of studies have reported chronic elevation in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in skeletal muscle fibers or in cultured myotubes from DMD patients and mdx mice. Two of the pathways that might be involved in the calcium overload are store operated channels (SOC) and stretch activated channels (SAC). Earlier results suggest that both channels are controlled, at least partly, by the Ca^{2+} -independent form of phospholipase A2 (iPLA₂) (Boittin et al., 2006). In the present study, we investigated the effect of doxorubicin (Dox), a chemotherapeutic agent reported to inhibit iPLA₂ (Swift et al., 2007), on the activity of iPLA₂ and the consequences that this might have on Ca^{2+} handling in both C2C12 and EDL mdx myotubes. PED-6, a fluorescent probe, was used to determine PLA₂ activity while ⁴⁵Ca²⁺ influx experiments were used to determine the effect of Dox on Ca^{2+} handling. Dox inhibited PLA₂ in clinically relevant concentrations (1–30 μM), reduced basal ⁴⁵Ca²⁺ entry down to 70% of control values, blocked the entry through SAC almost completely but had minimal effects on SOC in the tested concentrations. Further investigations are ongoing to better understand the mechanism of Dox on cellular Ca^{2+} homeostasis.

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P3.47

Administration of Losartan improves skeletal muscle repair in mice with sarcopenia

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Critical loss of muscle mass is observed in various systemic conditions as well as in the physiological process of aging (sarcopenia). This often not only increases morbidity and mortality, but also increases the incidence of pathologic fractures, functional deterioration and institutionalization. The molecular mechanisms underlying sarcopenia are only poorly understood, but it has recently been demonstrated that an increase in TGF-β signaling contributes to impaired satellite cell function and muscle repair in old skeletal muscle. We therefore evaluated whether antagonism of TGF-β signaling via administration of the angiotensin II type1 receptor blocker, Losartan, has any beneficial impact on the muscle repair process of aged mice *in vivo*. To assess muscle regeneration capacity, muscle injury was induced by administration of the snake venom cardiotoxin; skeletal muscles of aged mice were analyzed at 4 and 18 days after injury induction. We find that systemic TGF-β antagonism via Losartan significantly improves the muscle regeneration process in response to injury. Aged mice demonstrate an increase in regenerating muscle fibers at 4 days after injury induction. Furthermore, after 18 days, mice treated with Losartan exhibit significantly less fibrotic tissue formation and an overall more homogeneous appearance of regenerated skeletal muscle. Thus, pharmacological antagonism of TGF-β signaling may be a promising target for the management of age-related loss of muscle mass. Furthermore, Losartan mediated improvement of muscle regeneration may prove to reduce the rehabilitation time of frail individuals who have suffered muscular damage.

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