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Hypergravity from conception to adult stage: effects on contractile properties and skeletal muscle phenotype

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Summary

This study examined the effects of an elevation of the gravity factor (hypergravity – 2 *g*) on the molecular and functional characteristics of rat soleus and plantaris muscles. Long Evans rats were conceived, born and reared (CBR) continuously in hypergravity conditions until the age of 100 days. Whole muscle morphological parameters, Ca^{2+} activation characteristics from single skinned fibers, troponin (Tn) subunit and myosin heavy (MHC) and light (MLC) chains isoform compositions were examined in CBR and control muscles from age-paired terrestrial rats. Decreases in body and muscle mass in soleus and plantaris muscles were observed and associated, in the soleus, with a decrease in fiber diameter. The specific force of CBR soleus fibers was increased, and correlated with the elevation of Ca^{2+} affinity. This was accompanied by slow-to-slower TnC and TnI isoform transitions and a rearrangement in TnT fast isoform content. The MHC transformations of the soleus after hypergravity were associated with the up (down)-regulation of the MHCI (MHCIIa) mRNA isoforms. The MLC2 phosphorylation state remained unchanged in the soleus muscle. The results suggested that the gravity factor could interact with rat muscle development and that hypergravity experiments could provide good tools for the study of myofibrillar protein plasticity and their associated pathways of regulation.

Key words: 2G-centrifugation, skinned fiber, rat, soleus, plantaris, contractile and regulatory protein, electrophoresis, RT-PCR.

Introduction

It is now clear that nervous discharge patterns govern the acquisition of muscle definitive phenotype and that modified gravitational forces affect muscle development (Vrbova and Wareham, 1972; Butler-Brown and Whalen, 1984; Lomo, 1989; Picquet et al., 1998). To date, changes in gravitational environment have been studied extensively in real or simulated microgravity conditions (for a review, see Thomason and Booth, 1990). Under these conditions, slow extensor muscles presented an atrophy, a decrease in generated forces and a general slow-to-fast phenotype transformation (Gardetto et al., 1989; Musacchia et al., 1990; Stevens et al., 1993; Fauteck and Kandarian, 1995). The changes were particularly marked on the myosin heavy chains (MHC) and light chains (MLC) (Stevens et al., 1999a,b, 2000) as well as on the T, C and I subunits of troponin (Kischel et al., 2001; Bastide et al., 2002; Stevens et al., 2002). Moreover, these modifications were associated with a decrease in Ca^{2+} affinity of the contractile system.

Less is known about the adaptation of the muscular system under hypergravity (HG) conditions, particularly in terms of contractile protein isoform transitions, and/or Ca^{2+} activation properties. Most studies have examined rats born in normal gravity and placed as adults into an HG environment (Amtmann and Dyama, 1976; Roy et al., 1996; Chi et al., 1998; Stevens et al., 2003). For instance, adult rats exposed for 14 days (Roy et al., 1996) or 19 days in HG (Stevens et al., 2003) led to partial slow-to-fast transformation of the slow soleus phenotype without alteration of the developed forces. The phenotype transformation was characterized by an increase in fast isoform contents of TnC and TnI in relation to a higher cooperativity along the thin filament (steeper Tension/pCa relationship), whereas fast muscle was not (plantaris) or less (gastrocnemius) modified.

Few experiments have reported the effects of hypergravity on rats reared in HG. Martin (1980) has shown an elevation in slow oxidative fibers in soleus and plantaris muscles of 30-day old rats centrifuged at 2 ***g*** for 2 weeks. In the rectus capitis muscle (responsible for head posture) of rats conceived, born

and reared until the seventh postnatal day at 1.8 ***g*** centrifugation, Martrette et al. (1998) showed an increase in slow and perinatal MHC isoform expressions. In a recent study on rats reared in HG (Picquet et al., 2002), we showed that the slow soleus muscle studied *in situ* presented an increase in maximal and twitch tensions and was changed into a slower type, for both contractile parameters and MHC isoform content, while the fast plantaris was less affected.

Therefore the results obtained under lifelong conditions of hypergravity, when compared to both microgravity and hypergravity applied to adults, appeared opposite in terms of generated forces and MHC isoform transitions. This led us to characterize the effects of HG on Long Evans rats conceived, born and reared in hypergravity (2 ***g***) conditions, in order to understand how a complete life in HG could affect the generated force in relation to the muscle protein transformations. Two muscles were studied: the soleus muscle (a predominantly slow ankle extensor) and its fast agonist, the plantaris.

For this purpose, the different objectives of this work were: (1) analysis, at the cellular level, of the Ca^{2+} activation characteristics in relation to the forces developed by single fibers, in order to complete the study of Picquet et al. (2002), who showed, at the whole muscle level, a reduction of the absolute twitch and maximal tetanic forces, as well as a decrease in fiber cross sectional area after HG. (2) To study more deeply phenotype acquisition in muscles continuously exposed to 2 ***g***. The analysis in Picquet et al. (2002) was limited to the MHC, so to obtain a more complete understanding of the isoform transitions occurring after HG, we studied the three subunits of the troponin molecule (TnC, TnT and TnI, contributing to the regulation of Ca^{2+} activation properties) and the set of MLC, especially the regulatory MLC2, known to modulate the Ca^{2+} sensitivity of the contractile proteins by its phosphorylation state (Sweeney and Stull, 1996). Slow-to-slower MHC changes in contractile properties have already been described in CBR soleus muscle *in situ* (Picquet et al., 2002), so a study of MLC2 phosphorylation could contribute to our knowledge of the regulation of this phenotypical modification. Indeed, we have recently reported an increase of MLC2 phosphorylation associated with slow-to-fast transitions induced by hindlimb unloading in rat soleus muscle (Bozzo et al., 2003). Thus, we hypothesize that the slow-to-slower characteristics acquired after HG were associated with a decrease in MLC2 phosphorylation. (3) To estimate by reverse transcription–polymerase chain reaction (RT-PCR) analyses, the gene regulation of the MHC slow-to-slower transition observed previously (Picquet et al., 2002).

Materials and methods

Biological material

Experiments were performed on male Long Evans rats *Rattus norvegicus* Janvier aged 100 days divided into two groups: control rats (CONT, N=14) and rats subjected to hypergravity – chronic centrifugation (N=15) as previously described (Gustave Dit Duflos et al., 2000). Briefly, the latter rats were derived from couples placed in centrifugation apparatus, but only females remained until weaning. Male rats descending from different littermates were studied and named CBR because they were Conceived, Born and Reared until 100 days old in hypergravity. The CONT rats were reared under the same conditions as the CBR rats, i.e. the same room, same dark:light cycle (12 h:12 h), and the same temperature. CONT and CBR animals were anaesthetised by intraperitoneal injection of pentobarbital sodium (30 mg kg^{-1}) and weighed. For each group, soleus and plantaris muscles were dissected and weighed. After biopsy, the animals were killed with a lethal dose of pentobarbital sodium. One half of each muscle was skinned as previously described (Mounier et al., 1989) to study the contractile properties. The skinned muscles were stored at –20°C in a 50/50 glycerol/skinning solution (see below), containing protease inhibitor leupeptin (10 μg ml^{-1}), known to prevent loss of contractile proteins and to preserve the fiber tension (Reiser et al., 1988). The other half was frozen in liquid N_2 and stored at –80°C for electrophoretic study of protein composition.

The experiments and the maintenance conditions of the animals were approved by both the Agricultural and Forest Ministry and National Education Ministry (authorization 5900996).

Hypergravity conditions

Hypergravity was obtained in the same centrifugation apparatus as the one previously used by Picquet et al. (2002) and described by Gustave Dit Duflos et al. (2000). The centrifuge consisted of a velocity-controlled DC motor located in the vertical axis of the apparatus and driving two horizontal cross-arms (length 165 cm) at constant rotation speed. Four free-swinging gondolas were jointed at the four extremities of the horizontal arms, 76.5 cm away from the axis of rotation; the gondolas were equipped with lights that reproduced a 12 h:12 h light:dark cycle, and standard home cages for rats. One gondola carried a video system used to observe animal birth and behavior. During centrifugation, the gondolas were tilted at a constant angle of 60° depending on the centripetal acceleration for 2 ***g*** hypergravity environment. Counterclockwise rotations were achieved at constant velocities of 3.81 rad s^{-1}. Given the mass and the inertia of the gondolas, including the home cages and the rats, these angular velocities led to a 2 ***g*** resultant force whose direction was always similar to that exerted in normal gravity (directed dorso–ventrally, orthogonal to the antero–posterior axis of the animal).

Solutions

All reagents were provided by Sigma (St Louis, USA). The composition of all the solutions was calculated as previously described (Mounier et al., 1989). Final ionic strength was 200 mmol l^{-1}. Activating solutions contained MOPS, 10 mmol l^{-1}, potassium propionate (KProp, 185 mmol l^{-1}),

magnesium acetate (MgAc, 2.5 mmol l^{-1}) and various concentrations of free Ca^{2+} from $CaCO_3$, buffered with EGTA and added in proportions to obtain different pCa values (6.8–4.2, where pCa=–log[Ca^{2+}]). The pSr (pSr=–log[Sr^{2+}]) solutions were similar to the pCa solutions but with free Sr^{2+} from $SrCl_2$. Relaxing solution (R) was identical to the skinning solution and composed of 10 mmol l^{-1} MOPS, 170 mmol l^{-1} KProp, 2.5 mmol l^{-1} MgAc and 5 mmol l^{-1} EGTA. pH was adjusted to 7.0 and ATP at 2.5 mmol l^{-1} was added to each solution. Before applications of the Ca^{2+} or Sr^{2+} solutions, each fiber was bathed for 15 min in 2% Brij 58 (polyoxyethylene 20 cetyl ether) under constant stirring. The non-ionic Brij 58 detergent irreversibly eliminated the ability of the SR of skinned muscles to sequester and release Ca^{2+}, without altering the actomyosin system.

Force measurement and recording

The experiments were carried out in a thermostatically controlled room (19±1°C). Fibers were mounted in an experimental chamber and connected to a strain-gauge (Ford 10, World Precision Instruments, Aston, UK). A micrometer allowed fiber diameter measurements. The resting sarcomere length (*SL*) was determined by diffraction using a Helium/Neon laser (Spectra Physics, Carlsbad, USA). For soleus as well as for plantaris muscle fibers, the *SL* was set to 2.6 μm (120% of resting *SL*) to allow optimal isometric tension development upon ionic activation.

Single fibers were first immersed in a pCa 4.2 solution to measure the initial force. Next, they were checked for their strontium reactivity by successive exposures to pSr solutions: submaximal steady state tensions obtained in pSr 5.8, 5.4, 5.0, 4.6, were normalized to maximal Sr^{2+} activated tension (pSr 3.4), in order to deduce the half maximal activation (Sr^{2+}-affinity or pSr_{50}) by strontium from the linear part of the Tension–pSr (T–pSr relationship). Fibers were then activated with various pCa (from 6.8 to 4.2 for all fibers). The steady state submaximal tensions P were expressed as a percentage of the maximal tension P_0 (induced by the saturating pCa 4.2 solution), and reported as Tension-pCa (T–pCa) relationships. Fiber type was determined, using the difference between Ca^{2+} and Sr^{2+} activation characteristics for fast and slow fibers, the fast muscle fibers being less sensitive to Sr^{2+} than slow fibers (Kerrick et al., 1980). Fiber type was therefore determined by establishing the Δ value or difference between the respective Ca^{2+} and Sr^{2+}-affinity criteria, pCa_{50} and pSr_{50} (Ca^{2+} and Sr^{2+} concentration needed to elicit 50% of P_0). Typically, the Δ value of slow fiber is <0.30 pCa units, and ≥1.00 pCa units in fast fibers. Two other important parameters were deduced from the T–pCa relationships: the threshold for activation by Ca^{2+}, reflecting the sensitivity of the contractile system, and the steepness of the T–pCa curve, corresponding to the cooperativity between the different regulatory proteins within the thin filament. Fast type fibers could also be distinguished from slow type ones by a higher Ca^{2+} threshold (lower pCa value) and a steeper T–pCa curve, pCa_{50} not usually being significantly different (Stevens et al., 1993). The steepness of the T–pCa curve was determined by the Hill coefficient n_H, calculated according to the following equation: $P/P_0=([Ca^{2+}]/K)^{nH}/\{1+([Ca^{2+}]/K)^{nH}\}$, where P/P_0 is the normalized tension and K is the apparent dissociation constant ($pK=-\log K=pCa_{50}$).

Mono-dimensional electrophoresis for troponin and MHC analyses

Muscles stored at –80°C were pulverized under liquid N_2. Half the powder was dissolved in a buffer consisting of Tris-HCl 125 mmol l^{-1}, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 10% glycerol with 0.02% Bromophenol Blue. As previously described (Kischel et al., 2001), the different isoforms of the three troponin subunits were separated on a one-dimensional 10%–20% gradient gel and identified by immunoblotting (see next section).

As already described (Toursel et al., 2000), the MHC composition was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4.5% stacking gel and on a 7.5% separating gel. Electrophoresis was for 20 h at 12°C (180 V constant, 13 mA/gel). After the gel run, the MHC gel slabs were silver stained. The relative proportions of MHC isoforms in each sample was determined by integrating densitometry software (GS-700 Imaging Densitometer, Biorad, Ivry s/Seine, France). At least two independent measurements were performed on each sample. They were always quite similar and the mean value is reported.

Immunoblotting

Electrotransfer was performed on a 0.2 μm nitrocellulose sheet (Advantec MFS, Pleasanton, CA, USA). The membranes were blocked with a phosphate-buffered saline solution (PBS, pH 7.4) containing 5% non-fat dry milk and 0.2% sodium azide. All the membranes were incubated overnight with each primary antibody. As previously described (Stevens et al., 2002), a monoclonal antibody (5C5 from Sigma, specific to α sarcomeric actin) allowed actin signal recognition, serving as internal control. For TnT, the fast isoforms were identified with the JLT-12 monoclonal antibody from Sigma; the slow TnT isoforms were detected using a polyclonal antibody previously characterized and provided by Pette and collaborators (Härtner et al., 1989). For TnC, both slow and fast isoforms (50–50% recognition) were identified by another polyclonal antibody provided by Pette and collaborators (Härtner and Pette, 1990). For TnI, slow and fast isoforms were identified by two separate polyclonal antibodies also provided by Härtner and Pette (1990). Previous studies from our team (Stevens et al., 2003), however, have shown that each TnI antibody (slow or fast) recognized each TnI isoform (slow or fast), respectively, and with the same affinity. The primary antigen–antibody complexes were detected by extravidin peroxidase and biotinylated conjugate antibodies against mouse or guinea pig IgGs (Sigma). The signals were visualized by an enhanced chemiluminescence (ECL) kit (Amersham, Bucks, UK). Signal intensities were evaluated by integrating densitometry. At least two independent measurements were performed on each sample (averaged value reported).

The bound antibodies could be removed by an incubation at 50°C for 40 min with occasional stirring in a stripping buffer (100 mmol l^{-1} 2-mercaptoethanol, 2% SDS, 62.5 mmol l^{-1} Tris-HCl, pH 6.7). The membrane was washed twice in PBS at room temperature and the success of the stripping was tested by incubating the membrane with the secondary antibodies corresponding to the previously tested antibodies and ECL detection. Immunodetection of the other proteins was then performed as described above. To ensure that there was no muscle protein loss during incubation of the nitrocellulose membrane in the stripping buffer, the membrane was reincubated at the end of the experiments with the first used antibody and the intensities of the signals compared. No significant difference between the signals was measured.

Two-dimensional electrophoresis for MLC analysis

The other half of the muscle powder was used to extract myofibrillar protein for MLC analysis by 2-D gel electrophoresis. Myofibrillar proteins were extracted from 7–10 mg of dry muscle powder as described previously (Toursel et al., 2000), washed first with a solution containing 6.3 mmol l^{-1} EDTA (pH 7), pepstatin 0.1%, phenylmethylsulfonylfluoride (PMSF) 1%, and then with a second solution containing KCl 50 mmol l^{-1}, pepstatin 0.1%, PMSF 1%. The myofibrillar proteins were resuspended in 500 µl of milliQ-filtered water and their concentration determined by a protein assay kit (Dc Protein Assay, Bio-Rad) to prepare samples having a final quantity of 50 µg. Then, the proteins were precipitated for 2 h with acetone (8 v/v), followed by centrifugation for 1 h at 13 000 ***g***. The pellet was dissolved in Laemmli solution (Laemmli, 1970) for SDS-PAGE or in rehydration buffer for 2-D gel electrophoresis.

Proteins were separated by two-dimensional (2-D) gel electrophoresis using a procedure similar to those previously described by Morano et al. (1988), Gonzalez et al. (2002) and Bozzo et al. (2003). For the first dimension or isoelectric focusing (IEF), proteins were solubilized in 8 mol l^{-1} urea, 2% Chaps, 0.01 mol l^{-1} dithitreitol (DTT) and a 2% carrier ampholites (Amersham) buffer, and then separated using the Ettan IPGphor Isoelectric Focusing System (Amersham) on 3.5% acrylamide strips with immobilized pH gradients (47) (Amersham). Strips were rehydrated at 50 V for 12 h and proteins focused under the following voltage conditions: 500 V for 1 h, 500–1000 V for 1 h, 8000–100 000 V h^{-1}. Temperature was kept constant at 20°C. After reduction with 6 mol l^{-1} urea, 30% glycerol, Tris-HCl 0.375 mol l^{-1}, pH 8.8, 2% DTT and alkylation using the same buffer with additional 2.5% iodoacetamide, the strips were embedded in 4% polyacrylamide stacking gel and the proteins separated by SDS-PAGE on a 12% polyacrylamide gel for 8 h at 150 V at low temperature (4°C). Following electrophoresis, gels were silver stained. The positions of slow and fast isoforms of MLC on 2-D gels were determined according to their isoelectric point in the first IEF dimension, and to markers of appropriate molecular mass in the second dimension (Bozzo et al., 2003). All two-dimensional gels were digitized with an Epson 1650 scanner at a resolution of 200 dpi. The spots were analyzed densitometrically determining BAP (Brightness Area Product) with a constant threshold after black/white inversion using Adobe Photoshop Software (Bozzo et al., 2003).

Statistical analysis

All the data are reported as means ± S.E.M. The statistical significance of the difference between means was determined using the Student's *t*-test. Differences at or above 95% confidence level were considered significant ($P<0.05$).

Results

Body and muscle mass

The results are reported in Table 1. At the end of the 100 days in hypergravity conditions, the mean body mass (BM) of CBR rats was about 40% lower than that of CONT. Soleus and plantaris CBR muscles exhibited muscle wet mass (MWM) reductions of 42 and 46%, respectively, when compared to CONT. However, the MWM/BM ratio was not different between CONT and CBR in both soleus and plantaris muscles. Moreover, hypergravity induced, in soleus, a decrease of about 10% in diameter measured on isolated skinned fibers, while no change was found for plantaris fibers. Maximal tension (P_0) developed by isolated fibers and expressed per cross sectional

Table 1. *Morphological and force parameters after hypergravity*

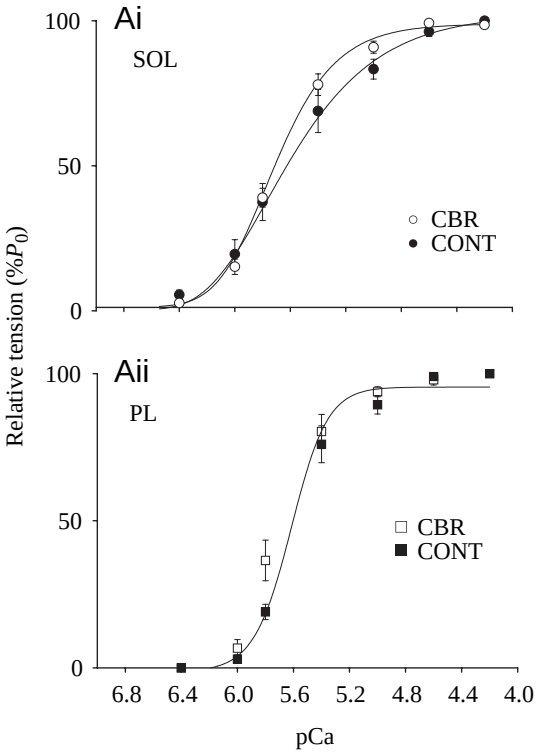
	Body mass (BM) (g)[a]	Muscle	Muscle wet mass (MWM) (g)[a]	MWM/BM (mg/g)[a]	Fiber diameter (µm)	P_0 (kN m^{-2})
CONT	370±15 (14)	SOL	198.71±19.97 (7)	0.54±0.03 (7)	84.72±2.78 (9)	65.80±7.34 (9)
		PL	430.86±18.31 (7)	1.15±0.05 (7)	76.67±3.21 (6)	151.89±25.93 (6)
CBR	219±22* (15)	SOL	114.76±13.98* (15)	0.53±0.07 (15)	77.27±1.95* (22)	99.92±10.71* (22)
		PL	231.75±47.50* (8)	1.04±0.14 (8)	72.35±2.23 (17)	119.48±13.77 (17)

Values are means ± S.E.M. (*N*).

CONT, control rats; CBR, rats conceived, born and reared in hypergravity (2 ***g***); P_0, relative maximal tension, expressed per cross sectional area of skinned fibers from SOL (soleus muscle) and PL (plantaris muscle).

[a]Data transposed from Picquet et al. (2002).

*Significantly different from CONT.



Bi

SOL	pCa_{thr}	pCa_{50}	n_H
CONT (*N*=8)	6.65±0.12	5.61±0.09*	1.94±0.32*
CBR (*N*=21)	6.42±0.07	5.75±0.02*	3.01±0.19*

Bii

PL	pCa_{thr}	pCa_{50}	n_H
CONT (*N*=6)	6.02±0.05	5.60±0.06	4.20±0.34
CBR (*N*=13)	6.10±0.03	5.68±0.05	4.46±0.55

Fig. 1. Tension–pCa relationships of SOL (soleus) and PL (plantaris) fibers in control (CONT) rats and rats conceived, born and reared in hypergravity (CBR). (A) CONT and CBR T–pCa curves of slow SOL fibers (Ai) and fast PL fibers (Aii). (Bi,ii) Parametric values derived from T–pCa curves of the fibers analyzed in A. pCa_{thr}, threshold for Ca^{2+} activation; pCa_{50}, pCa at which tension is half maximum; n_H, Hill coefficient. Values are means ± S.E.M. Curves were fitted according to the Hill equation. *Significantly different from CONT (P<0.05).

area (kN m^{-2}) was significantly increased (by a factor 1.5) for CBR soleus and not modified for CBR plantaris muscles.

Ca^{2+} activation properties of isolated skinned fibers

The T–pCa curves and their derived parameters are illustrated for soleus and plantaris in Fig. 1. We chose to test only slow fibers in both CONT and CBR soleus muscles (Fig. 1Ai,Bi). Slow fibers of CBR rat soleus exhibited a mean T–pCa curve shifted to lower calcium concentrations when compared to CONT. The Ca^{2+} affinity parameter (pCa_{50}) was increased by 0.14 pCa units and the steepness (n_H value) of the curve was elevated by 55% in CBR fibers. The calcium sensitivity (pCa threshold) was not affected.

Ca^{2+} activation properties of fast plantaris fibers were not altered after hypergravity conditions (Fig. 1Aii,Bii, bottom). All characteristics of CBR fibers from plantaris presented values identical to those of CONT ones.

Protein expression of troponin isoforms

The expression patterns of the different isoforms of the three subunits of troponin are illustrated in Fig. 2A and their relative expression reported in Fig. 2B. All the troponin subunit identifications were performed using specific antibodies against slow and fast Tn isoforms (see Materials and methods). As described in Fig. 2A,B (top), TnT protein is composed, in control soleus from Long Evans rats, of four fast isoforms:

Table 2. *Distribution of total slow and fast myosin light chain (MLC) isoforms and relative repartition of MLC2 isoforms in CONT and CBR soleus muscles*

	% Distribution							
	MLC1		MLC2		MLC3	MCL2 isoforms (% repartition)		
	Slow	Fast	Slow	Fast	Fast	MLC2s	MLC2s1	MLC2f
CONT	84±6	16±6	92±4	8±4	100±0	79±2	21±2	100±0
CBR	96±3*	4±3*	95±3	5±3	100±0	80±1	20±1	100±0

CONT, control rats; CBR, rats conceived, born and reared in hypergravity (2 ***g***).
Slow or fast MLC1, MLC2 and MLC3 isoforms expressed as % of the total (slow+fast) MLC1, MLC2 or MLC3 isoforms.
Each MLC2 isoform (s, s1 and f) expressed as % of the total MLC2 isoforms.
*Significantly different from CONT.

TnT1f, 2f, 3f and 4f and three slow isoforms: TnT1s, 2s and 3s. In CBR rats, the relative distribution of fast isoforms was modified with an upregulation of TnT3f (20% increase) and a downregulation of TnT1f (50% decrease) when compared to CONT soleus. However, no change was observed for TnTs isoforms. In CBR soleus muscles (Fig. 2A,B), the relative percentages of slow (fast) TnI isoforms were increased (decreased) by ~20%. The same effects (amount and direction) were observed for TnC isoforms.

No variation in Tn (T, I and C) isoform expression in the plantaris was seen in hypergravity conditions.

Protein expression of MLC isoforms and MLC2 phosphorylation

Since no difference was found for the three Tn subunits in plantaris, the effects of hypergravity on MLC expression and MLC2 phosphorylation were tested only in soleus muscles. The CONT soleus expressed predominantly the slow isoforms of MLC, slow MLC1 (84%) and slow MLC2 (92%), when compared to their respective fast counterparts, fast MLC1 (16%) and fast MLC2 (8%) (Table 2). The MLC3 was detected in the 2-D gels but at very low levels. In CBR soleus, the transitions could be seen only for MLC1 isoform distribution

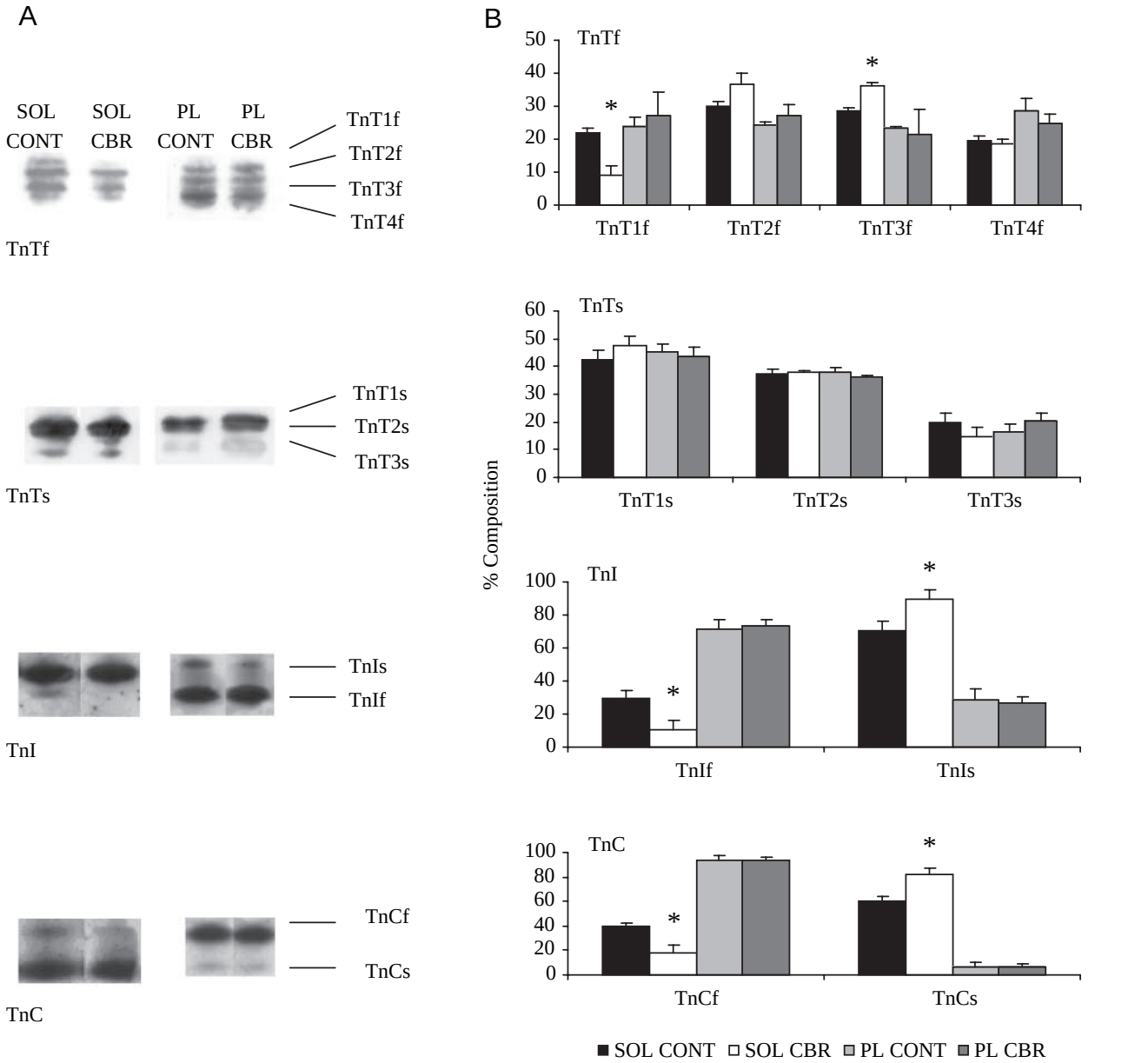


Fig. 2. Expression of troponin (Tn) protein isoforms. (A) ECL profiles of soleus (SOL) and plantaris (PL) in control (CONT) rats and rats conceived, born and reared in hypergravity (CBR), obtained by immunoblotting after SDS-PAGE in a 10%–20% gel. (B) Relative amounts of Tn (T, I, C) isoforms electrophoretically separated. For TnT subunit, each slow or fast isoform was expressed as a percentage within the total amount of slow (TnTs) or fast (TnTf) isoforms, respectively. For TnI and TnC subunits, each slow (or fast) isoform was expressed in % of the total amount of (slow + fast) isoforms. *Significantly different from CONT (P<0.05).

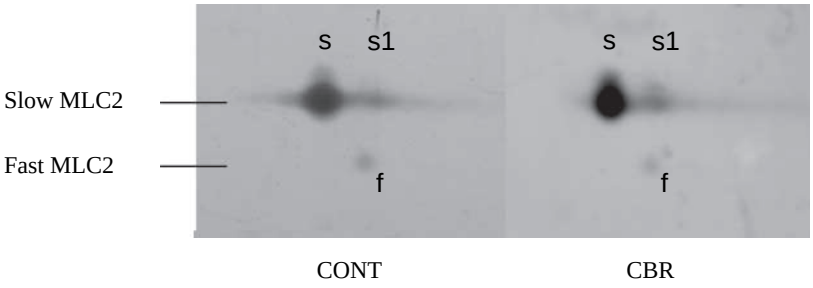


Fig. 3. Spots of MLC2 isoforms as they appeared in 2-D gel electrophoresis of soleus muscles from control rats (CONT) and rats conceived, born and reared in hypergravity (CBR). s, s1 and f, see text for explanation.

and were characterized by a 12% increase in slow MLC1 isoform, with a concomitant decrease in fast MLC1, which remained slightly persistent (4%). No change was observed in MLC2 or MLC3 expression.

Analysis of MLC2 phosphorylation after hypergravity is also shown in Table 2 and in Fig. 3, which illustrates a 2-D gel and indicates the position of the different MLC2 spots (s, s1 and f). As described previously (Bozzo et al., 2003), the positions of slow and fast MLC2 isoforms were confirmed by immunoblotting (data not shown). In the CONT soleus, slow MLC2 was the predominant regulatory light chain and appeared, in fact, to be separated into two spots: MLC2s (as described above) and another spot with a more acidic isoelectric point, MLC2s1. MLC2s and MLC2s1 have previously been identified by alkaline phosphatase experiments (Bozzo et al., 2003) as unphosphorylated and phosphorylated spots, respectively. Hypergravity conditions did not modify the extent of phosphorylation of the MLC2s1 isoform in soleus muscles since there was no change in MLC2 spot unphosphorylated/phosphorylated distribution in CBR soleus compared to control muscles (Fig. 3). For the fast MLC2 isoforms, both

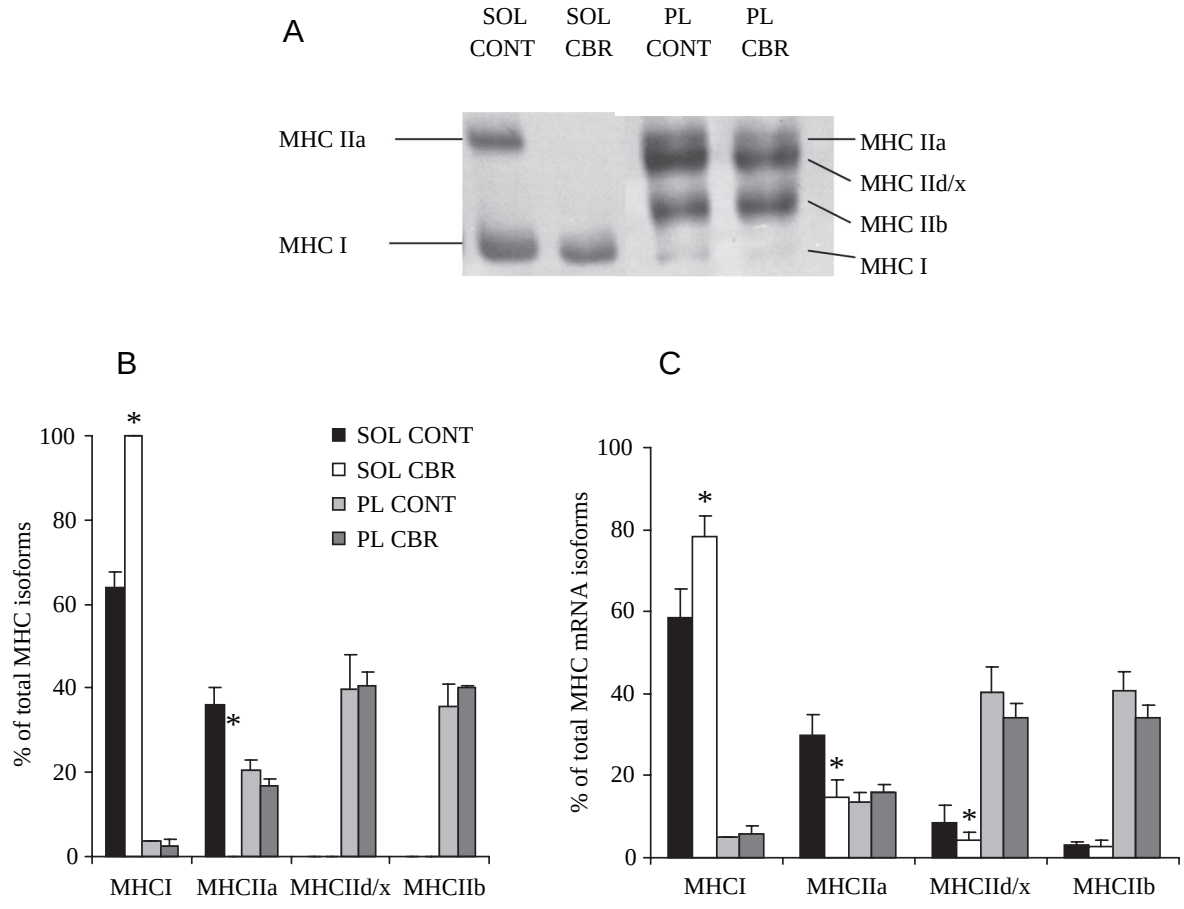


Fig. 4. Expression patterns of MHC protein and mRNA isoforms (A) SDS-PAGE (7.5%) of MHC isoforms of soleus (SOL) and plantaris (PL) from CONT and CBR rats. (B) Relative amounts of MHC protein isoforms in SOL and PL muscles from CONT and CBR rats. Each isoform was expressed as a percentage of total MHC isoforms. (C) Relative amounts of MHC mRNA isoforms in SOL and PL muscles from CONT and CBR rats. The absolute MHC mRNA isoforms were obtained by RT–PCR and expressed as percentages of total mRNA isoforms. *Significantly different from CONT (P<0.05). Values are means ± S.E.M.; invisible error bars are compressed in the histogram thickness.

CONT and CBR soleus presented one single spot, the unphosphorylated MLC2f.

Protein and mRNA expressions of MHC isoforms

The changes in MHC isoform composition are described in Fig. 4. These data were obtained from the same animals as Picquet et al. (2002). HG conditions had important and significant effects on the MHC isoform repartition in soleus muscle. At the protein level (Fig. 4B), two MHC isoforms were expressed in the CONT soleus: the predominant slow MHCI isoform (64%) and the fast MHCIIa isoform (36%). As previously reported (Picquet et al., 2002), the slow MHCI was significantly increased in CBR muscles since its expression reached 100% of total MHC content, while MHCIIa was no longer expressed. RT–PCR analysis of MHC mRNA isoform content of the SOL muscle demonstrated that the four MHC mRNA isoforms (MHCI, IIa, IId/x and IIb) were present in CONT and CBR soleus muscles (Fig. 4C). The slow MHCI isoform was increased compared to CONT (~78% *versus* 58%) while fast MHCIIa and IId/x mRNA isoforms were decreased by 50% in CBR rats. MHCIIb mRNA isoform, present at very low amounts in CONT SOL (~3%), was not affected.

At both protein and mRNA levels, the CONT plantaris muscle expressed the four MHC isoforms previously described, but with a predominance of the fast isoforms in the order MHCIId/x ⩾MHCIIb >MHCIIa >MHCI. The protein and mRNA isoform distributions were not modified in CBR plantaris muscles.

Discussion

The aim of this work was to analyze contractile properties and hindlimb muscle phenotype transformations of rats conceived, born and reared in hypergravity induced by 2 ***g***-centrifugation. The results showed a decrease in body mass and soleus weight associated with a decrease in fiber diameter. Increased specific maximal force correlated to increases in Ca^{2+} affinity and cooperativity within the thin ˇfilament, were amply demonstrated. They were accompanied by slow-to-slower phenotype transitions of the contractile and regulatory proteins. Except for muscle mass (~46% decrease), no change was observed in plantaris muscle in hypergravity.

Body and muscle mass, diameter and maximal tension of muscle fibers

As already described and discussed by Picquet et al. (2002), CBR rats exhibited body and muscle masses lower than those of control muscles. These authors concluded that the decrease in muscle mass was correlated with the decrease in body mass, which could, in turn, be related to a slowing down in the growth of the rats conceived and reared in hypergravity. They also observed a significant decline in the absolute CSA of the slow soleus fibers (–30%) and the fast plantaris (–17%) ones. These observations were confirmed by our measurements on skinned fiber diameters, the difference in the amplitude (–10% in soleus and –6% in plantaris fiber diameters) of the decreases being possibly explained by the different techniques of analysis and muscle fiber treatments (skinning) used.

Our results showed that the maximal tensions (kN m^{-2}) were increased by 34% in CBR soleus fibers. This also corresponded with the results of Picquet et al. (2002), obtained at the whole soleus muscle level. Here, the increase in specific maximal force could be linked either to an increased number of cross-bridges or to an increase in force output per cross-bridge. The first hypothesis would suppose an elevated synthesis/degradation ratio of proteins in CBR soleus fibers, a fact that was not supported by the observed decrease in fiber diameter. Some studies have suggested that hypergravity did not induce changes in protein synthesis (Almurshed and Grunewald, 2000), but had a general sparing effect on muscle proteins in rats that were not gaining body mass, thus permitting them to maintain muscle protein levels (Roy et al., 1996). Another explanation for the increase in the number of cross-bridges would be a modification in the myofilament lattice spacing, already proposed for microgravity conditions (Fitts et al., 2000). The second hypothesis, i.e. the elevated maximal tension P_0 (kN m^{-2}) explained by an increase in force per cross-bridge, was in good agreement with the observed increase in Ca^{2+} affinity in CBR soleus fibers (see below).

For the plantaris muscle, the absence of alteration in specific force P_0 (kN m^{-2}) in hypergravity, agreed with the lack of changes in calcium activation characteristics (especially in Ca^{2+} affinity parameter).

Contractile properties and troponin isoform transitions

This is the first time that changes in body and muscle growth of rats conceived, born and reared in hypergravity, have been shown to be accompanied by modifications in calcium activation characteristics of soleus but not plantaris muscle fibers. These changes consisted principally of a higher Ca^{2+} affinity and a higher cooperativity between proteins of the thin filament, indicating that CBR soleus fibers were able to produce more efficient contractions. These effects were specific to CBR animals (including animal gestation, birth and development), since only slight modifications (and in the opposite direction) have been reported for adult rats placed in hypergravity for a period of 19 days (Stevens et al., 2003). In our study, the electrophoretic analyses showed an increase in the relative proportions of slow isoforms of troponin subunits. It is well known that the isoform type of the three Tn subunits present in the muscle fibers can influence the shape of the T–pCa curve (Schiaffino and Reggiani, 1996). Here, the isoform composition of the three subunits (T, C and I) was affected. Thus, upregulation of TnC and TnI slow isoforms *versus* downregulation of the fast ones could explain very well the higher Ca^{2+} affinity observed in CBR soleus fibers. Indeed, slow fibers generally exhibit higher Ca^{2+} affinity than fast ones (Mounier et al., 1989; Schiaffino and Reggiani, 1996). Changes at the TnT level were less marked: the slow TnT content was not modified and a rearrangement within the fast TnT isoforms occurred, consisting of an increased relative level of TnT3f at the expense of TnT1f, one of the two isoforms (with TnT4f)

more representative of fast muscles. Thus, we suggested that the higher cooperativity observed in CBR soleus fibers might be linked to the rearrangement in TnT fast isoforms. Tropomyosin transformations can also be envisaged to explain the modifications in Ca^{2+} affinity (Schachat et al., 1987).

MLC and MLC2 isoform transitions

In our study, MLC slow-to-slower changes were seen only for the slow (fast) MLC1 isoform, which was significantly increased (decreased) in hypergravity. Thus, transitions at the total MLC level were less marked than for MHC (see below) and troponin isoforms. Such lesser effects of environmental conditions on MLC changes have already been described elsewhere (Ingalls et al., 1996; Stevens et al., 2000).

Another means of regulation, the phosphorylation of the MLC2 isoform, has previously been described as positively correlated with a slow-to-fast phenotype transformation in slow muscles (Bozzo et al., 2003). Here, the slow-to-slower phenotype transitions induced in CBR soleus were not accompanied by variations in MLC2 phosphorylation. One could have expected a decrease in phosphorylation. A possible explanation for these latter results could be the low level of MLC2 phosphorylation existing in the slow soleus muscle. Moreover, decreases (increases) in MLC2 phosphorylation have generally been associated with declined (elevated) Ca^{2+} affinities (Sweeney and Stull, 1986).

This was not the case in this study, since we did not demonstrate any change in phosphorylation paralleling the decrease in Ca^{2+} affinity in CBR soleus. As previously described by Bozzo et al. (2003), in unloaded soleus, MLC2 phosphorylation was increased alongside a decrease in Ca^{2+} affinity (Gardetto et al., 1989; Stevens et al., 1993). These observations led us to suggest that modifications in Ca^{2+} affinity induced by changes in the gravity factor could not be directly explained by variations in MLC2 phosphorylation.

MHC isoform transitions

The most important biochemical changes in CBR soleus reported here were characterized by slow-to-slower transitions, observed at the MHC level. Our results are in agreement with those previously described by Martin (1980) on Sprague-Dawley rats submitted to centrifugation at 30 days of age, but not with studies on rats placed in hypergravity for 2 weeks as adults (Roy et al., 1996; Stevens et al., 2003). This suggested that a nervous factor could be participating in the observed transformations. Indeed, Krasnov et al. (1992) have demonstrated in soleus from rats grown in hypergravity that the volumes of the bodies, nucleus and nucleolus in motoneurons from the spinal cord at the level of lumbar enlargement were increased. These authors thus suggested a higher functional motoneuron activity, associated with an elevated content of slow and intermediate muscle fibers.

In our conditions and as previously described (Picquet et al., 2002), the CBR soleus muscle only expressed the slow MHCI isoform. A total disappearance of all fast isoform expression, more precisely of MHCIIa, was observed. These changes were in agreement with those occurring at the mRNA level, i.e. a shift from MHCIIa mRNA (~30% in CONT/~15% in CBR) to MHCI mRNA (~60% in CONT/~80% in CBR). However, MHCIIa mRNA was still present in CBR muscles even though the protein was no longer expressed, suggesting altered translational and/or post-translational regulation during hypergravity.

In plantaris muscle, we also found no changes in maximal tension or contractile characteristics in MHC and troponin isoform compositions. The lack of changes in MHC isoforms described in this study does not conflict with the increased number of hybrid fibers observed by Picquet et al. (2002). Indeed, a rearrangement in the MHC isoform distribution among the different fibers would not necessary lead to a change in MHC content at the whole muscle level. Therefore, as was the case in real or simulated microgravity conditions, hypergravity at 2 ***g*** preferentially affected the phenotype of slow hindlimb extensor muscles.

In conclusion, the present study performed on rats conceived, born and reared in hypergravity, reported effects on soleus muscle that are contrary to those observed in adult rats exposed to hypergravity. Indeed, in the latter (Stevens et al., 2003), only a few modifications were shown, consisting of a decrease in Ca^{2+} affinity, and a slight slow-to-fast transition of TnC and I isoforms; MHC and TnT isoforms were unaffected. In contrast, the present study on CBR rats highlights an increase in Ca^{2+} affinity and important changes in the expression of myofibrillar proteins: troponins present slow-to-slower transitions and MHC isoform pattern are reduced to the single expression of the slow MHCI isoform. In this context, other periods of hypergravity initiation could be further examined with the aim of understanding which critical steps of rat gestation, birth and/or development influence the transformations induced by changes in gravity factor. Indeed, since the muscles in our study were removed at the adult stage, we could not determine whether or not the MHC plasticity under centrifugation went through remodelling steps including modified early-expressed myosin isoforms, like embryonic or neonatal MHC (Martrette et al., 1998).

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