Impaired structural and functional regeneration of skeletal muscles from β2-adrenoceptor knockout mice

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Abstract

Aims: β2-adrenergic stimulation causes beneficial effects on structure and function of regenerating muscles; thus, the β2-adrenoceptor may play an important role in the muscle regenerative process. Here, we investigated the role of the β2-adrenoceptor in skeletal muscle regeneration.

Methods: Tibialis anterior (TA) muscles from β2-adrenoceptor knockout (β2KO) mice were cryolesioned and analysed after 1, 3, 10 and 21 days. The role of β2-adrenoceptor on regenerating muscles was assessed through the analysis of morphological and contractile aspects, M1 and M2 macrophage profile, cAMP content, and activation of TGF-β signalling elements.

Results: Regenerating muscles from β2KO mice showed decreased calibre of regenerating myofibres and reduced muscle contractile function at 10 days when compared with those from wild type. The increase in cAMP content in muscles at 10 days post-cryolesion was attenuated in the absence of the β2-adrenoceptor. Furthermore, there was an increase in inflammation and in the number of macrophages in regenerating muscles lacking the β2-adrenoceptor at 3 and 10 days, a predominance of M1 macrophage phenotype, a decrease in TβR-I/Smad2/3 activation, and in the Smad4 expression at 3 days, while akirin1 expression increased at 10 days in muscles from β2KO mice when compared to those from wild type.

Conclusions: Our results suggest that the β2-adrenoceptor contributes to the regulation of the initial phases of muscle regeneration, especially in the control of macrophage recruitment in regenerating muscle through activation of TβR-I/Smad2/3 and reduction in akirin1 expression. These findings have implications for the future development of better therapeutic approaches to prevent or treat muscle injuries.

Keywords: akirin1, contraction, macrophage, skeletal muscle regeneration, Smad, β2-adrenoceptor.
and secrete pro-inflammatory cytokines, such as interleukin 1β (IL-1β), and inducible nitric oxide synthase (iNOS) (Locati et al. 2013, Mantovani et al. 2013). Subsequently, there is a shift in phenotype from M1 to alternatively activated M2 macrophages, which express the mannose receptor (CD206) and secrete anti-inflammatory cytokines, such as arginase-1 and found in inflammatory zone 1 (Fizz1) (Locati et al. 2013, Mantovani et al. 2013). Growth and repair of myofibres after injury are mediated by a resident population of mononuclear myogenic precursor cells, the satellite cells (SCs), which are small progenitor cells located between the basal lamina and the sarcolemma of individual muscle fibres (Ferrari et al. 1998). Following muscle injury, these cells divide and give rise to the SC progeny, the myoblasts, which fuse to each other to repair or replace damaged fibres (Blau et al. 1993, Boldrin et al. 2010).

Although the morphological aspects of skeletal muscle regeneration are well known, the molecular players involved in this process have not been fully elucidated. Accordingly, the signalling pathways that regulate protein turnover are strongly modulated over the course of this process (Drummond et al. 2009, Miyabara et al. 2010). The phosphoinositide 3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) pathway and the ubiquitin proteasome system exert an essential role in skeletal muscle mass recovery after damage (Stipanuk 2007, Vary & Lynch 2007, Ge et al. 2009, Miyabara et al. 2010). Likewise, members of the transforming growth factor β (TGF-β) superfamily, such as myostatin, TGF-β1, TGF-β1 receptors (TGF-β-Rs), and their intracellular regulatory protein-receptor-regulated Smad2 and 3, and co-Smad, Smad4 are important to the success of muscle regenerative response (Drouglett et al. 2010, Zhu et al. 2011). Furthermore, more recent studies have suggested the involvement of other factors, such as Mustn1 and aki-rin1, in the regulation of skeletal muscle regeneration, considering that Mustn1 is highly expressed during myoblast fusion (Krause et al. 2013), and aki-rin1 is strongly induced in activated satellite cells and macrophages (Salerno et al. 2009). The β2-adrenoceptor may play an important role in muscle regeneration, as several studies have shown that β2-adrenergic stimulation, through muscle treatment with β2-adrenergic agonists, increases the size and strength recovery of regenerating myofibres (Beitzel et al. 2004, 2007, Bricout et al. 2004, Ryall et al. 2008, Conte et al. 2012).

Skeletal muscle contains a significant proportion of β2-adrenoceptors, of which the β2-subtype is the most prevalent (90%) (Kim et al. 1991). β2-adrenoceptors belong to the guanine nucleotide-binding G protein-coupled receptor (GPCR) family. The classical β2-adrenoceptor signalling pathway through Gz-AC-cAMP activation is, at least in part, responsible for the β2-adrenoceptor-mediated hypertrophy in the skeletal muscle (Navegantes et al. 2000, 2002, Hinkle et al. 2002). Once activated, cAMP can interact with the exchange protein directly activated by cAMP (EPAC) through the small GTPase Rap1, which may trigger the PI3K/AKT/mTOR signalling pathway, resulting in increased protein synthesis (Lynch & Ryall 2008). In addition, activated cAMP can bind to protein kinase A, which phosphorylates calpastatin, leading to the inhibition of the proteolytic activity of the calpain enzymes (Lynch & Ryall 2008). Furthermore, the Gβγ dimer seems to activate the PI3K/AKT/mTOR signalling pathway, thereby contributing to muscle hypertrophy (Kline et al. 2007, Lynch & Ryall 2008).

Recently we have shown that β2-adrenergic stimulation, through formoterol treatment, is able to increase the size of regenerating myofibres in young and old rats through the activation of mTOR (Conte et al. 2012). In fact, previous studies have demonstrated that the β2-adrenergic agonists clenbuterol and fenoterol improved the recovery of myofibre size in regenerating skeletal muscles after transplantation (Roberts & McGeachie 1992) and muscle injury (Beitzel et al. 2004, Bricout et al. 2004). Additionally, other studies have indicated that β2-adrenoceptor stimulation contributes to the increase in satellite cell proliferation and differentiation (Roberts & McGeachie 1992, 1994, Spurlock et al. 2006), enrichment of vascular repair (Roberts & McGeachie 1994), decrease in inflammation and a more efficient restoration of the extracellular matrix (Pullar et al. 2008, Zhang et al. 2010). However, the role of the β2-adrenoceptor in skeletal muscle regenerative process and its importance to the muscle contractile recovery after injury is still unknown.

Considering that β2-adrenergic stimulation enhances the structural and functional repair of regenerating muscle, we hypothesized that the β2-adrenoceptor plays an important role in the skeletal muscle regenerative process. Therefore, this study aimed to investigate the role of the β2-adrenoceptor in histological, molecular and functional aspects of skeletal muscle regeneration of β2-adrenoceptor knockout (β2KO) mice. To attain this goal, we evaluated morphological and muscle contractile aspects, M1 and M2 macrophage profile, cAMP content, activation of TGF-β signalling elements [TGF-β receptor I (TbR-I) and Smad2/3], and aki-rin1 and Smad4 expression in regenerating TA muscles from β2KO mice.
Methods

All protocols used in this study were in accordance with Good Publishing Practice in Physiology (Persson 2013).

Animals

A cohort of male congenic Friend virus B-type (FVB) mouse strain lacking β2-adrenoceptor (β2KO) (Chruscienski et al. 1999) and their age-matched FVB wild type (WT) controls at 2 months of age were used in this study. Mice were maintained in standard housing conditions (Conte et al. 2012).

Experimental design

In this study, one TA muscle (left hind limb) from each animal was subjected to cryolesion (Cryo group), and the contralateral TA muscle (right hind limb) was used as control (CL group). WT and β2KO animals were randomized into four groups for the histological experiments (n = 6, each group). Both WT and β2KO animals were subjected to cryolesion and were evaluated at 1, 3, 10 and 21 days post-cryolesion respectively. Contralateral muscles were designated as intact controls (CL group). In addition, distinct animals were used for the muscle contraction and cAMP quantification experiments and were evaluated at post-cryolesion day 10 (Conte et al. 2012).

Based on Pereira et al. (2014), animals were anesthetized and had the superior surface of the TA muscle entirely exposed. The cryolesion procedure was performed according to Conte et al. (2012), using the iron rod (flat end) dimensions of 2 × 9 mm and bringing it into contact with the muscle twice (i.e., in the two longitudinal halves of the muscle).

Histology

Animals were killed by decapitation, after which their left and right TA muscles were removed, weighed and stored according to Pereira et al. (2014). Muscle cross sections (10-μm-thick) obtained from the TA middle-bellies were stained with an aqueous solution of toluidine blue and borax (Miyabara et al. 2004). Necrotic myofibres were localized by histochemical detection of lysosomal acid phosphatase activity (Gomori lead method) (Bancroft 1996, Miyabara et al. 2010).

The stained sections were analysed in a Nikon Eclipse light microscope (PCM2000; Nikon, New York, NY, USA), and figures were prepared using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

Cyclic AMP (cAMP) quantification

cAMP quantification was performed according to Conte et al. (2012).

Antibodies for immunostaining

The primary antibodies used for immunostaining were (1) mouse monoclonal anti-MAC387 (macrophage) (1 : 200; cat# ab22506; Abcam, Cambridge, MA, USA), (2) rabbit polyclonal anti-TGF-β receptor I phospho S165 (cat# ab112095, 1 : 250; Abcam) and (3) rabbit monoclonal anti-Smad2/3 (cat# 8685; 1 : 200; Cell Signaling Technology, Danvers, MA, USA). The corresponding secondary antibodies used were (1) goat anti-mouse IgG-fluorescein isothiocyanate (FITC) (1 : 200; cat# 115-095-166; Jackson ImmunoResearch Laboratories, Baltimore, PA, USA); and (2) goat anti-rabbit IgG-rhodamine (1 : 200; cat# ab6718; Abcam).

Immunostaining

Muscle cross sections were immunostained according to Pereira et al. (2014). Photomicrographs of the stained sections were obtained using a fluorescence microscope equipped with a 20× magnification objective lens (Nikon, Eclipse E1000, ACT-1 version 2.2 or Observer D1; Zeiss; Jena, Germany), and with FITC, Rhodamine and fura filters.

Antibodies for Western blotting

The primary antibodies used for Western blotting were (1) rabbit polyclonal anti-Smad4 (1 : 1000; cat# 9515; Cell Signaling Technology), (2) rabbit polyclonal anti-akirin1 (1 : 1000; cat# ab77075; Abcam) and (3) rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1 : 2000; cat# ABS16; Millipore, Temecula, CA, USA). The corresponding secondary antibody used for all Western blots was peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1 : 10 000; cat# 111-035-003; Jackson Immuno Research Laboratories).

Western blot analysis

Western blot analysis was executed according to Conte et al. (2012). Densitometry analysis was performed based on Pereira et al. (2014).

Gene expression analysis

Reverse transcription. Total RNA was isolated from muscle samples using Trizol reagent (Invitrogen,
 Primer design. The following primer sequences were used: Fizz-1: 5'-GAGACCATAGAGATTCTCTGG A-3' forward, 5'-CACACCAGTACGATC-3' reverse; Arginase-1: 5'-CAATGAAGGCTGCTGTT GT-3' forward, 5'-GGTGAGGATCCACCTGCTTCC-3' reverse; iNOS: 5'-CTGAGGACCTTGGAATCG-3' forward, 5'-CTGACACCCAAAATGTCTC-3' reverse; IL-1β: 5'-CCCAAGATGAGGGCTGCTTCC-3' forward, 5'-GGATGGGCTCTTCTTCAAAGATG-3' reverse and TFIID: 5'-TTAGGCCTGGAAAGGTGTTA CG-3' forward, 5'-CCTCCAAACAGATGGAAACAT TCT-3' reverse. Primer sequences for Fizz-1, Arginase-1, iNOS were obtained from Villalta et al. (2011) and primer sequences for IL-1β and the transcription factor IID (TFIID) were obtained from Acharyya et al. (2007) and Silva et al. (2012) respectively. The primers were synthesized from Exteend Biotecnologia, Brazil.

Real-time PCR. Real-time PCR was carried out on an ABI Prism 7300 Sequence detection system (QuantStudioTM 12K Flex Real-Time PCR System; Applied Biosystems, Foster City, CA, USA) according to Aoki et al. (2009). The transcription factor TFIID was used as the housekeeping gene. Expression values are reported as fold difference relative to the expression of the control housekeeping gene. Expression values are reported as 2^-ΔΔCt. The WT Cryo 1d group was used as control when no amplification was detected in WT CL group.

In addition to 3 and 10 days post-injury, the time point of day 1 was included in the gene expression analysis of the M1 and M2 macrophage markers, considering that M1 macrophages invade the wound site at 1 day post-damage (Turner & Badylak 2012).

Flow cytometry

Muscle samples underwent two enzymatic dissociations and, subsequently, the cells were filtered with a 40-μm cell strainer (BD Biosciences, San Jose, CA, USA) (Cheung et al. 2012). Based on Larocca et al. (2013), the filtrate was centrifuged at 600 g for 15 min and cells were resuspended in lyses buffer for erythrocytes removal. Cell pellets were resuspended in PBS supplemented with 2% foetal bovine serum and stained with the following antibodies at 1: 100 dilution for 30 min at 4 °C in the dark: APC-Cy7-conjugated anti-CD11b, PE-conjugated anti-CD86 (for validation of M1 polarization) and APC-conjugated anti-CD206 (for validation of M2 polarization). At least 50 000 cells were acquired with BD FACSCanto II using the FACSDIVA software (BD Biosciences). The data analysis was performed with FLOWJo software (Tree Star, Ashland, OR, USA). A gate for macrophages was set based on forward scatter (FSC) and CD11b positive. From this population, gates for CD86 and CD206 positive cells were created. Results were present as the percentage of positively stained cells within the cell population (macrophage gates) or macrophage population (CD86 and CD206 gates). The results are representative of 3 distinct experiments.

Mechanical measurements

Mechanical measurements in TA muscles were obtained according to Pereira et al. (2014). Frequencies of isolated twitches, maximum tetanic contraction and tetanus during fatigue protocol were 0.2, 200 and 100 Hz respectively.

Quantitative and morphometric analyses

ImagePro PLUS software (Media Cybernetics, Silver Spring, MD, USA) was used for the quantitative and morphometric analyses.

Myofibre CSA was manually measured, and it was obtained from all regenerating myofibres with centralized nuclei in muscles analysed on post-cryolesion day 10 and 21 (Pereira et al. 2014). Necrotic myofibres in three whole cross sections for each muscle (n = 3) stained with acid phosphatase and analysed on post-cryolesion day 1 were counted and expressed as the percentage of total myofibres.

Regenerating myofibres were counted and expressed as the percentage of total myofibres according to Conte et al. (2012).

The areas of inflammation in the muscle cross sections stained with toluidine blue and analysed on post-cryolesion days 3 and 10 were measured and expressed as the percentage of the whole muscle cross section (n = 4 per group).

Macrophages were quantified based on Pereira et al. (2014).

The TβR-I positive area and the number of nuclei positive for Smad2/3 were obtained from the injured muscle area of the whole muscle cross sections analysed at 3 days post-cryolesion (n = 3, each group). Subsequently, the injured area and the whole muscle cross-section area were measured. The TβR-I positive area was expressed as the percentage of the entire muscle cross section, and the incidence of Smad2/3 positive nuclei was expressed as the number of Smad2/3 positive nuclei per square millimetre.

Statistical analysis

Multiple comparisons of mean values were performed according to Conte et al. (2012). The unpaired t-test
was used for comparisons between two groups (Miyabara et al. 2010). For all comparisons, \( P \leq 0.05 \) was considered significant.

**Results**

**Body and muscle weights**

The body weight of WT mice was not altered over the course of 21 days (Table 1), whereas the body weight of \( \beta_2 \text{KO} \) mice increased 8% at 10 days and 12% at 21 days (\( P \leq 0.05 \); Table 1).

At 1 day post-cryolesion, muscle weight of both WT and \( \beta_2 \text{KO} \) mice increased, when compared with the respective contralateral group (47 and 37% respectively; \( P \leq 0.05 \); Table 1), but muscle weight of \( \beta_2 \text{KO} \) mice increased 13% less than that of WT mice, in the same period (\( P \leq 0.05 \); Table 1). On post-cryolesion day 3, muscle weight of both WT and \( \beta_2 \text{KO} \) mice decreased, when compared with muscles on post-cryolesion day 1 (28 and 27%, respectively, \( P \leq 0.05 \); Table 1). However, muscle weight was unchanged when compared to the respective contralateral groups (Table 1).

When analysed at 10 days, muscle weight of WT mice decreased by 24%, when compared to its respective contralateral group (\( P \leq 0.05 \); Table 1), whereas muscle weight of \( \beta_2 \text{KO} \) mice remained unchanged, when compared with muscles on post-cryolesion day 1 (28 and 27%, respectively, \( P \leq 0.05 \); Table 1). At 10 days post-cryolesion, muscle weight of \( \beta_2 \text{KO} \) mice decreased, when compared with the respective contralateral group (\( P \leq 0.05 \); Table 1). On post-cryolesion day 3, muscle weight of both WT and \( \beta_2 \text{KO} \) mice decreased, when compared with muscles on post-cryolesion day 1 (28 and 27%, respectively, \( P \leq 0.05 \); Table 1). However, muscle weight was unchanged when compared to the respective contralateral groups (Table 1).

When analysed at 10 days, muscle weight of WT mice decreased by 24%, when compared to its respective contralateral group (\( P \leq 0.05 \); Table 1), whereas muscle weight of \( \beta_2 \text{KO} \) mice remained unchanged in \( \beta_2 \text{KO} \) mice. On post-cryolesion day 21, muscle weight of WT mice increased by 35%, when compared with that on post-cryolesion day 10 (\( P \leq 0.05 \); Table 1), until it reached a value similar to the respective contralateral group (Table 1). By contrast, muscle weight of \( \beta_2 \text{KO} \) mice remained unchanged at 10 and 21 days post-cryolesion (Table 1).

**Histology**

Histological cross sections of TA muscle samples collected on post-cryolesion days 1, 3, 10 and 21 were stained with toluidine blue and analysed by light microscopy. Intact contralateral muscles from both WT and \( \beta_2 \text{KO} \) mice exhibited polygonal fibres with peripheral nuclei, which is typical of normal tissue structure (Fig. 1).

Contralateral muscles from \( \beta_2 \text{KO} \) mice had slightly smaller myofibre CSAs when compared to muscles from WT mice, that is, 59% of myofibres were smaller than 2000 \( \mu \text{m}^2 \) in the contralateral \( \beta_2 \text{KO} \) group, whereas 54% of myofibres were smaller than 2000 \( \mu \text{m}^2 \) in the contralateral WT group (\( P \leq 0.05 \); Fig. 1).

On post-cryolesion day 1, tissue damage in both WT and \( \beta_2 \text{KO} \) mice was evident in freeze-injured TA muscles, as confirmed by the presence of hypercontracted fibres, inflammatory cell infiltration and clear spaces between the cells, indicating myonecrosis, tissue disruption and oedema (Fig. 1). In addition, the percentage of necrotic myofibres in muscles analysed on post-cryolesion day 1 from both WT and \( \beta_2 \text{KO} \) mice was similar (43\( \pm \)9 and 44\( \pm \)11%, respectively, Fig. 1).

At 3 days post-cryolesion, a small inflammatory area was evident in muscles from both WT and \( \beta_2 \text{KO} \) mice (18\( \pm \)3 and 25\( \pm \)4% of the entire muscle cross section, respectively, Fig. 1), with a tendency of increase in muscles from \( \beta_2 \text{KO} \) mice compared to those from WT mice (Fig. 1). Hypercontractured myofibres were also observed in \( \beta_2 \text{KO} \) mice (Fig. 1), suggesting the persistence of acute signs of injury.

At 10 days post-cryolesion, 69.5% of myofibres containing central nuclei from WT mice were smaller than 1200 \( \mu \text{m}^2 \), against only 15% in the contralateral group (\( P \leq 0.05 \); Fig. 1). Additionally, the regenerating muscles from WT group showed split fibres. At the same time point, regenerating myofibres with central nuclei from \( \beta_2 \text{KO} \) mice were smaller than those from the contralateral group (74% smaller than those from the contralateral group).

**Table 1** Comparison of body (BW, g) and muscle weight (MW, mg) from cryolesioned groups from wild-type (WT) and \( \beta_2 \)-adrenoceptor knockout (\( \beta_2 \text{KO} \)) mice.

| Groups     | C         | Cryo 1d | Cryo 3d | Cryo 10d | Cryo 21d |
|------------|-----------|---------|---------|----------|----------|
| BW (g)     |           |         |         |          |          |
| WT         | 27.4 (1.3)| 27.5 (1.3)| 28.1 (0.8)| 28.3 (1.4)| 29.1 (1.4) |
| \( \beta_2 \text{KO} \)| 26.6 (1.2)| 26.7 (1.2)| 27.4 (1.2)| 28.7 (1.8)*| 29.8 (2.0)* |

| Groups     | CL 1d | Cryo 1d | CL 3d | Cryo 3d | CL 10d | Cryo 10d | CL 21d | Cryo 21d |
|------------|-------|---------|-------|---------|--------|----------|--------|----------|
| MW (mg)    |       |         |       |         |        |          |        |          |
| WT         | 36.6 (0.7)| 53.8 (1.1)*| 36.6 (1.9)| 38.6 (3.2)*| 35.8 (0.8)| 27.2 (2.0) ab | 38.9 (1.8)| 36.6 (1.3) ab |
| \( \beta_2 \text{KO} \)| 34.3 (1.0)| 46.9 (2.5)* b*| 39.1 (1.0)| 34.4 (3.9)*| 39.0 (4.1)| 34.0 (4.2) ab | 39.1 (1.1)| 38.2 (2.3) b* |

Values are expressed as means and SD; \( n = 6 \). ANOVA followed by the Bonferroni’s test for multiple comparisons was applied to test differences among the various groups. BW, body weight; MW, muscle weight; C, intact control, Cryo 1d, Cryo 3d, Cryo 10d, and Cryo 21d, cryolesioned and analysed after 1, 3, 10 and 21 days; CL, intact muscle from the contralateral leg, respectively.

\*\( P \leq 0.05 \) vs. matched control or matched CL.

\#\( P \leq 0.05 \) vs. matched group from WT; \#\( P \leq 0.05 \) vs. Cryo 1d; \#\( P \leq 0.05 \) vs. Cryo 3d; \#\( P \leq 0.05 \) vs. Cryo 10d.
Figure 1  (a) Representative toluidine blue stained tibialis anterior (TA) muscle cross sections of WT and β2KO mice. (a) WT contralateral group; (b) KO contralateral group; (c) WT, 1 day post-cryolesion; (d) β2KO, 1 day post-cryolesion; (e) WT, 3 days post-cryolesion; (f) β2KO, 3 days post-cryolesion; (g) WT, 10 days post-cryolesion; (h) β2KO, 10 days post-cryolesion; (i) WT, 21 days post-cryolesion; (j) β2KO, 21 days post-cryolesion. Arrows, hypercontracted fibres; asterisks, inflammatory cell infiltration; arrow head, cells with centralized nuclei; small arrows, split fibres. Bar: 50 μm. (b) Comparison of percentage of necrotic myofibres in muscles from WT and β2KO mice at 1 day post-cryolesion (% of total myofibres from whole muscle CSA). Data are expressed as mean and SD; n = 3. (unpaired t-test). (c) Comparison of percentage of inflammatory area in muscles from WT and β2KO mice at 3 and 10 days post-cryolesion (% of whole muscle CSA). Data are expressed as mean and SD; n = 4. (analysis of variance followed by Bonferroni’s test for multiple comparisons). # P ≤ 0.05 vs. WT mice; *p ≤ 0.05 vs. Cryo 3d. (d) Frequency distribution of myofibre cross-sectional area (CSA) in contralateral and cryolesioned muscles from WT and β2KO mice analysed at 10 and 21 days post-cryolesion; n = 5. Kolmogorov–Smirnov test was applied in order to test differences between groups. (e) Comparison of percentage of centrally nucleated myofibres in muscles from WT and β2KO mice at 10 and 21 days post-cryolesion (% of total myofibres from whole muscle CSA). Data are expressed as mean and SD; n = 3. (analysis of variance followed by Bonferroni’s test for multiple comparisons).
were smaller than 1200 \( \mu m^2 \) in the \( \beta_2 \)-KO group, against 21\% in the contralateral group, \( P \leq 0.05 \); Fig. 1) and from WT mice (54\% in \( \beta_2 \)-KO vs. 62\% in WT, with CSA values between 400 and 1200 \( \mu m^2 \) respectively; \( P \leq 0.05 \); Fig. 1). Also, muscles from the \( \beta_2 \)-KO group exhibited typical signs of acute lesion, such as hypercontracted fibres and inflammatory infiltration, which was larger compared to that from the WT group (254\%, \( P \leq 0.05 \); Fig. 1). In addition, the area of inflammation increased in muscles from the \( \beta_2 \)-KO group analysed on post-cryolesion day 10, when compared to those analysed on post-cryolesion day 3 (47\%, \( P \leq 0.05 \); Fig. 1). However, the area of inflammation was unchanged in the WT group (Fig. 1).

On post-cryolesion day 21, TA muscles from freeze-injured WT mice were completely regenerated, as evidenced by the classical signs of regeneration (Karpati et al. 1988), that is, restored myofibres with centralized nuclei, CSA values similar to contralateral muscles, as well as split fibres (Fig. 1). At this same time point, regenerating myofibres from \( \beta_2 \)-KO mice were larger than those from the Cryo 10d group (74\% were larger than 1200 \( \mu m^2 \) in the \( \beta_2 \)-KO group, \( P \leq 0.05 \); Fig. 1) and from WT mice (74\% were larger than 1200 \( \mu m^2 \) in the \( \beta_2 \)-KO group, \( P \leq 0.05 \); Fig. 1). In addition, cryolesioned muscles analysed at 21 days post-injury from \( \beta_2 \)-KO mice had regenerating myofibres with centralized nuclei with similar calibre size, when compared to those from WT mice (Fig. 1).

The percentage of regenerating myofibres with centralized nuclei was unaltered in the groups analysed on post-cryolesion days 10 and 21 (Fig. 1).

**Total number of macrophages and muscle macrophage phenotype**

**Incidence of Mac387 positive macrophages.** At 3 and 10 days post-cryolesion, the number of macrophages in muscles from WT and \( \beta_2 \)-KO mice increased when compared to the respective controls (WT: 4978 and 3988\% respectively; \( \beta_2 \)-KO: 10 038 and 8572\% respectively; \( P \leq 0.05 \); Fig. 2). In addition, at 3 and 10 days post-cryolesion, the number of macrophages in muscles from \( \beta_2 \)-KO mice was 157 and 173\% higher than that observed in muscles from WT mice respectively (\( P \leq 0.05 \); Fig. 2).

**M1 and M2 associated genes.** Gene expression of IL-1\( \beta \) and arginase-1 did not change in all groups evaluated (Fig 3).

\( \beta_2 \)-adrenoceptor mRNA expression did not change in muscles from WT group evaluated at 1, 3 and 10 days post-cryolesion, when compared to the respective contralateral muscles (Fig 3). On day 1 post-cryolesion, iNOS mRNA expression in muscles from \( \beta_2 \)-KO group increased 705\% when compared to that of the contralateral muscles, and 1116\% when compared to that from WT group, evaluated in the same time point post-injury (\( P \leq 0.05 \); Fig. 3). By contrast, iNOS mRNA expression in muscles from \( \beta_2 \)-KO group decreased at 3 and 10 days post-cryolesion when compared to that obtained on day 1 post-cryolesion (89 and 82\% respectively; \( P \leq 0.05 \); Fig. 3).

Fizz-1 mRNA expression was unaltered in muscles from WT group in all time points analysed (Fig. 3). Fizz-1 mRNA expression increased in the contralateral muscles from \( \beta_2 \)-KO group, when compared to that from the contralateral muscles from WT group (1238\%, \( P \leq 0.05 \); Fig. 3). By contrast, Fizz-1 mRNA expression decreased in the injured muscles at 1, 3 and 10 days post-injury when compared to that obtained in the contralateral muscles from \( \beta_2 \)-KO group (96, 99 and 92\%, respectively, \( P \leq 0.05 \), Fig. 3).

**CD11b\(^{+ve} \), CD86\(^{+ve} \) and CD206\(^{+ve} \) cell population.** The CD11b\(^{+ve} \) population increased in regenerating muscles from both WT and \( \beta_2 \)-KO mice analysed at 3 and 10 days when compared to the respective controls (WT: 557 and 286\% respectively; \( \beta_2 \)-KO: 459 and 419\%, respectively, \( P \leq 0.05 \); Fig. 4), and this increase was more pronounced in muscles from \( \beta_2 \)-KO mice when analysed at 3 days (46\%, \( P \leq 0.05 \); Fig. 4), but not at 10 days.

The percentage of CD86\(^{+ve} \) cells in the CD11b\(^{+ve} \) population in muscles from WT and \( \beta_2 \)-KO mice analysed at 3 and 10 days did not change in any of the groups evaluated. However, the percentage of
CD86+ve cells tended to increase in the CD11b+ve population in regenerating muscles from β2KO mice, when compared to those from WT mice at the same time point evaluated (Fig. 4).

The percentage of CD206+ve cells in the CD11b+ve population in regenerating muscles from WT and β2KO mice analysed at 3 and 10 days decreased when compared to the respective contralateral muscles (WT: 96 and 50% respectively; P ≤ 0.05; β2KO: 94 and 47%, respectively, P ≤ 0.05; Fig. 4).

**Activation of TβR-I/Smad2/3**

With regard to the immunostaining against TβR-I, contralateral muscles from both WT and β2KO mice did not present immunopositivity for TβR-I (data not shown). The area positive for TβR-I in injured muscles from β2KO mice was 64% smaller than that from WT mice at the same time point evaluated (P ≤ 0.05; Fig. 5). Similarly, nuclei positive for Smad2/3 were not observed in contralateral muscles from either WT or β2KO mice (data not shown). Also, at 3 days post-cryolesion, 28% fewer nuclei were positive for Smad2/3 in muscles from β2KO mice, compared to muscles from WT mice at the same time point evaluated (P ≤ 0.05; Fig. 6).

**Smad4 and akirin1 expression in regenerating muscles**

At 3 days post-cryolesion, Smad4 expression decreased in injured muscles from WT and β2KO mice when compared to their controls (25 and 41% respectively; P ≤ 0.05; Fig. 7) and this decrease was more pronounced in muscles from β2KO mice when compared to those from WT group (32%; P ≤ 0.05; Fig. 7). At 10 days post-cryolesion, Smad4 expression decreased at the same rate in injured muscles from both WT and β2KO mice, when compared to their controls (29 and 25% respectively; P ≤ 0.05; Fig. 7). Moreover, the Smad4 expression in contralateral muscle from β2KO mice decreased when compared to contralateral muscle from WT group (24%; P ≤ 0.05; Fig. 7).

Akirin1 expression was not detected in contralateral muscles from either WT or β2KO groups (Fig. 7), but it was highly expressed at 10 days post-cryolesion in muscles from WT and β2KO mice when compared to their respective controls (1931 and 2387% respectively; P ≤ 0.05; Fig. 7). In addition, the akirin1 expression in injured muscle from β2KO mice assessed on post-cryolesion day 10 was more robust when compared with that in injured muscles from WT mice (95%; P ≤ 0.05; Fig. 7).
We investigated whether disruption of the β2-adrenoceptor gene target would decrease cAMP levels in contralateral and injured muscles and whether cryolesion by itself would change such levels in muscles from both WT and β2KO mice. No changes were observed in cAMP levels in contralateral muscles from WT and β2KO mice (Fig. 8). As expected, cryolesion alone induced a 228% increase in cAMP levels in muscles from WT mice after 10 days, when compared to their controls (P ≤ 0.05; Fig. 8). In addition, the increase in cAMP levels was attenuated in TA muscles from β2KO mice at the same time point, when compared to their controls (125%, P ≤ 0.05; Fig. 8).

**Maximum tetanic and specific forces, and resistance to fatigue**

The influence of the β2-adrenoceptor on the functional recovery of freeze-injured muscles was evaluated through the analysis of in vivo muscle contraction measurements on post-cryolesion day 10.

At the pre-fatigue tetanic contraction measurement, the absolute and specific force decreased by 34 and 43%, respectively, in the cryolesioned muscles from WT mice, when compared to the contralateral muscles from WT mice (P ≤ 0.05, Fig. 9). Likewise, the tetanic force and specific force decreased by 43 and 27%, respectively, in the injured muscles from β2KO mice, when compared to the contralateral muscles from β2KO mice (P ≤ 0.05, Fig. 9). Contralateral muscles...
from $\beta_2$KO mice showed a 17% decrease in absolute force and a 29% decrease in specific force, when compared to those from WT mice ($P \leq 0.05$, Fig. 9). Similarly, injured muscles from $\beta_2$KO mice showed 18% lower tetanic force and 17% lower specific force, compared to those from WT mice ($P \leq 0.05$, Fig. 9).

Regarding the post-fatigue tetanic and specific forces, we observed a reduction in tetanic and specific forces in injured TA muscles from WT and $\beta_2$KO mice when compared to their controls (WT mice: 42 and 40% respectively; $\beta_2$KO mice: 43 and 24% respectively; $P \leq 0.05$, Fig. 9). Similarly to the pre-fatigue tetanic contraction measurement, contralateral and injured muscles from $\beta_2$KO mice showed lower tetanic and specific force, when compared to those from WT mice (contralateral muscles: 24 and 34% decrease respectively; cryolesioned muscles: 26 and 16% decrease respectively; $P \leq 0.05$, Fig. 9).

The post-fatigue tetanic and post-fatigue specific forces decreased, when compared to the pre-fatigue tetanic and pre-fatigue specific forces, in both WT and $\beta_2$KO mice (contralateral muscles from WT mice: 11 and 9% decrease respectively; cryolesioned muscles from WT mice: 10 and 13% decrease respectively; contralateral muscles from $\beta_2$KO mice: 20 and 16% decrease respectively; cryolesioned muscles from $\beta_2$KO mice: 19 and 13% decrease respectively; $P \leq 0.05$, Fig. 9).

Tibialis anterior muscle strength was measured at four time points during the fatigue protocol (1st, 4th, 7th and 10th contractions). In contralateral muscles from both WT and $\beta_2$KO mice, there was a significant decrease.
loss of muscle strength beginning at the 4th contraction stimulus of the fatigue protocol (a 12 and 11% decrease in comparison to the 1st contraction respectively), and that difference persisted up to the 10th contraction \( (P \leq 0.05, \text{Fig. 9}) \). Also, muscle strength at the 1st contraction was significantly lower in the Cryo group than in the contralateral group from both WT and \( \beta_2 \)-KO mice (39 and 45%, respectively; \( P \leq 0.05, \text{Fig. 9} \)). In the Cryo group from WT and \( \beta_2 \)-KO mice, muscle strength was also significantly lower at the 4th, 7th and 10th contractions, in comparison with the 1st contraction (WT: 13, 18 and 77% respectively; \( \beta_2 \)-KO: 11, 19 and 25% respectively; \( P \leq 0.05, \text{Fig. 9} \)). In addition, strength in contralateral and cryolesioned muscles from \( \beta_2 \)-KO mice decreased when compared to the respective contralateral and cryolesioned muscles from WT mice at all time points (1st, 4th, 7th and 10th) evaluated (CL: 17, 16, 15 and 38% respectively; Cryo: 25, 24, 27 and 27% respectively; \( P \leq 0.05, \text{Fig. 9} \)).

**Discussion**

Skeletal muscle regeneration is a crucial process responsible for the recovery and maintenance of muscle mass and function. The present study indicated...
that the β2-adrenoceptor was an important player in this process, as mice lacking this receptor had structural and functional impairments in skeletal muscle regeneration. The time points of 1, 3, 10 and 21 days post-injury were assessed because they represent important phases of skeletal muscle adaptation to injury, including intense necrosis and oedema, inflammation and satellite cell proliferation, evident regeneration with the reestablishment of myofibril proteins, and restoration of the structural and functional features of the skeletal muscle respectively (Hawke & Garry 2001, Miyabara et al. 2006).

Initially, a gain in body weight was observed in β2KO mice compared to WT mice, suggesting that the β2-adrenoceptor contributed to the regulation of body weight. Notably, this finding is in line with previous studies indicating that β2-adrenergic stimulation causes lipolysis (Louis et al. 2000, Hinkle et al. 2002, Zhang et al. 2007, Lynch & Ryall 2008, Kim & Kong 2010, Kim et al. 2010) and aids in glucose uptake in brown adipocytes from rodents (Marette & Bukowiecki 1990, Shimizu et al. 1996, Chernogubova et al. 2004). The β2-adrenergic agonists are known to induce lipolysis through the activation of Gα-AC-cAMP followed by protein kinase A-mediated phosphorylation of adipose triglyceride lipase, hormone-sensitive-lipase and lipid-bound protein perilipin A; which result in increased hydrolysis of triacylglycerol, diacylglycerol and monoacylglycerol, and the release of free fatty acids from adipocytes (Holm 2003, Pagnon et al. 2012). Therefore, the absence of β2-adrenoceptor may contribute to fat mass accumulation.

With regard to muscle weight, it is interesting to note that β2KO mice gained less muscle weight at 1 day post-injury, compared to WT mice, which may
be related to a delay in the installation of oedema and inflammation that persisted for 10 days. In agreement with that, other studies have shown that β2-adrenoceptor stimulation induces a reduction in inflammation and a better remodelling of the extracellular matrix (Pullar et al. 2008, Zhang et al. 2010). Therefore, it is probable that the β2-adrenoceptor regulates the inflammatory process. In fact, there was a more pronounced increase in the area of inflammation and in the number of macrophages in regenerating muscles lacking the β2-adrenoceptor, which led us to investigate possible mechanisms involved in the recruitment of inflammatory cells during muscle regenerative process. In this respect, our data suggest that, in the absence of the β2-adrenoceptor, there might be an increase in the frequency of M1 macrophages in regenerating muscles, indicated by a robust increase in iNOS gene expression at 1 day post-injury and a tendency of increase in the number of CD86+ cells at 3 and 10 days post-injury, which could delay the shift in M1 to M2 macrophage phenotype, and consequently delay the muscle regenerative process. In fact, the shift in M1 to M2 macrophage phenotype is essential for the progression of myogenic differentiation and muscle regeneration (Tsujinaka et al. 1996, Szalay et al. 1997, Wang et al. 2014).

One possible mechanism involved in the shift of M1 to M2 phenotype through the β2-adrenoceptor is through cAMP production (Panina-Bordignon et al. 1997, Guereschi et al. 2013). Accordingly our results indicate that the increase in cAMP levels was attenuated in regenerating myofibres lacking the β2-adrenoceptor. However, the downstream signalling mechanisms are not well understood. Another possibility could involve the transactivation of TβR-I by the β2-adrenoceptor. More specifically, it is known that GPCRs are able to trans-activate serine/threonine kinase receptors (Burch et al. 2012), and that akirin1 is a target gene for serine/threonine kinase receptor stimulation (Marshall et al. 2008, Salerno et al. 2009, Dong et al. 2013). Therefore, we hypothesized that the absence of the β2-adrenoceptor might inhibit the transactivation of TβR-I, and subsequently inhibit its downstream targets, the Smad proteins, which could ultimately lead to increased akirin1 transcription. In fact, we observed a reduced activation of TβR-I and Smad2/3 and a decreased Smad4 expression in regenerating muscles lacking the β2-adrenoceptor at 3 days post-cryolesion. As a consequence, there was a more pronounced increase in the expression of akirin1, a macrophage chemoattractant, in regenerating muscles lacking the β2-adrenoceptor at 10 days post-cryolesion.

Akirin1 expression in macrophages, as well as its chemotactic properties in these cells (Salerno et al.
quent increase in \([\text{Ca}^{2+}]_i\) (Cairns et al. 2009). In addition, akirin1 has been shown to induce post-mitotic differentiation and myotube hypertrophy (Marshall et al. 2008), which is in line with our findings on the increased akirin1 expression at 10 days post-cryolesion. Therefore, a higher expression of akirin1 in regenerating muscles lacking the \(\beta_2\)-adrenoceptor could also serve as a compensatory mechanism, favouring the muscle regenerative response.

The increase in myofibre size during muscle regeneration seemed to be partially regulated by the \(\beta_2\)-adrenoceptor, as demonstrated by a moderate reduction in the calibre of regenerating myofibres from \(\beta_2\)-KO mice at 10 days post-cryolesion, when compared to those from WT mice. This result might be explained, to some extent, by the attenuated increase in cAMP levels in regenerating muscles lacking the \(\beta_2\)-adrenoceptor. Remarkably, this finding agreed with previous reports indicating that \(\beta_2\)-adrenergic stimulation by muscle treatment with \(\beta_2\)-agonists after injury led to an increase in both muscular cAMP levels and myofibre calibre (Roberts & McGecachie 1992, Beitzel et al. 2004, 2007, Bricout et al. 2004, Conte et al. 2012), and these effects could be reversed by the \(\beta_2\)-antagonist ICI-118551 (Zhong et al. 1996, McCormick et al. 2010).

With regard to the muscle contraction results, the decreased contractile function in muscles from \(\beta_2\)-KO mice may be due to impaired contractile mechanisms caused by the absence of the \(\beta_2\)-adrenoceptor, rather than to a higher susceptibility to muscle damage, as the \(\beta_2\)-KO mice had the number of necrotic myofibres similar to those from WT mice. In line with our data, a study indicated that the stimulation of the \(\text{Na}^+–\text{K}^+\) pump with the \(\beta_2\)-adrenoceptor agonist salbutamol, epinephrine, rat calcitonin gene-related peptide (rCGRP), or dibutyryl cAMP, improved force recovery by 40–90\% (Mikkelsen et al. 2006). In addition, it is known that the \(\beta_2\)-adrenergic agonist terbutaline potentiates peak twitch and tetanic force in rat soleus myofibres, which is related to an increase in intracellular free \(\text{Ca}^{2+}\) ([\(\text{Ca}^{2+}]_i\)) (Ha et al. 1999). Considering that this effect is consistent with a mechanism whereby terbutaline activates \(\beta_2\)-adrenoceptors leading to the protein kinase A-dependent phosphorylation of ryanodine receptor (Ha et al. 1999) with consequent increase in [\(\text{Ca}^{2+}]_i\), (Cairns et al. 1993) and that terbutaline’s effect on myofibre force is blocked by the \(\beta_2\)-adrenoceptor antagonist ICI 118551; the decreased absolute and specific tetanic forces found in the TA muscles lacking the \(\beta_2\)-adrenoceptor may occur due to an attenuation of protein kinase A-dependent phosphorylation of ryanodine receptor.

The lack of the \(\beta_2\)-adrenoceptor, combined with cryolesion, led to much less muscle force production, which might be a consequence of attenuated increase in [\(\text{Ca}^{2+}]_i\), associated with poor muscle mass recovery in regenerating myofibres under the absence of the \(\beta_2\)-adrenoceptor. Muscles lacking the \(\beta_2\)-adrenoceptor on post-cryolesion day 10 exhibited a larger area of inflammation and smaller-sized regenerating myofibres compared to those from WT mice. Therefore, it is reasonable to suggest that these muscles contained fewer myofibrillar components, thereby contributing to the reduction of force output. In line with the effects caused by the absence of \(\beta_2\)-adrenoceptors in regenerating skeletal muscle, we have demonstrated that the \(\beta_2\)-adrenergic stimulation through formoterol treatment is able to decrease the number of inflammatory cells in regenerating muscles, increase the size of regenerating myofibres, and consequently increase muscle force (Conte et al. 2012). In addition, previous studies have also claimed that \(\beta_2\)-adrenoceptor stimulation led to improved muscle force generation after injury (Beitzel et al. 2004, Ryall et al. 2008), which reinforces the hypothesis that the \(\beta_2\)-adrenoceptor has a functional importance in muscle regenerative process.

Since there was no difference in the myofibre CSA from \(\beta_2\)-KO and WT mice on post-cryolesion day 21, we did not measure muscle function in this time point. Considering that the structure and function of regenerating muscles from \(\beta_2\)-KO mice analysed on post-cryolesion day 21 were probably similar to their non-injured contralateral muscles, the \(\beta_2\)-adrenoceptor may be involved in the regulation of early phases of the muscle regenerative process, such as inflammation and recovery of myofibre size, and through unknown mechanisms, the delay in these early stages may be compensated.

In summary, this work demonstrates that the \(\beta_2\)-adrenoceptor contributes to early stages of muscle regeneration, being particularly involved in the regulation of macrophage recruitment to the regenerating skeletal muscle tissue, possibly through activation of \(\text{TjR-4}\) and Smad2/3 as well as a reduction in akirin1 expression. Therefore, the \(\beta_2\)-adrenoceptor may be an important clinical target for therapeutic interventions with pharmacological agents and gene therapy, with an aim to accelerate the initial phases following muscle injury, and consequently improve recovery and decrease the rehabilitation period in several conditions, such as sports injuries and the damage caused by surgical procedures, accident and frostbite.
Conflicts of interest

The authors declare that they have no conflict of interest.

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