Cardiotoxin-induced skeletal muscle injury elicits profound changes in anabolic and stress signaling, and muscle fiber type composition

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Abstract

To improve muscle healing upon injury, it is of importance to understand the interplay of key signaling pathways during muscle regeneration. To study this, mice were injected with cardiotoxin (CTX) or PBS in the Tibialis Anterior muscle and were sacrificed 2, 5 and 12 days upon injection. The time points represent different phases of the regeneration process, i.e. destruction, repair and remodeling, respectively. Two days upon CTX-injection, p-mTORC1 signaling and stress markers such as BiP and p-ERK1/2 were upregulated. Phospho-ERK1/2 and p-mTORC1 peaked at d5, while BiP expression decreased towards PBS levels. Phospho-FOXO decreased two and five days following CTX-injection, indicative of an increase in catabolic signaling. Furthermore, CTX-injection induced a shift in the fiber type composition, characterized by an initial loss in type IIa fibers at d2 and at d5. At d5, new type IIb fibers appeared, whereas type IIa fibers were recovered at d12. To conclude, CTX-injection severely affected key modulators of muscle metabolism and histology. These data provide useful information for the development of strategies that aim to improve muscle molecular signaling and thereby recovery.

Keywords: cardiotoxin, inflammation, muscle injury, muscle metabolism, muscle regeneration

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Introduction

Muscle injuries are a widely occurring event. Improper muscle regeneration might have long-term consequences, e.g. a decreased functional capacity. Therefore, many studies focus on strategies to improve the regeneration process. Since ethical and practical constrains limit investigating muscle injuries in humans, animal muscle injury models provide valuable insights in the regeneration process. The main advantages of these models, when compared to injuries in humans, involve a fast regeneration process (most of the muscle histology is repaired within 2 weeks), standardization of injury and feasibility to apply complex interventions, eventually resulting in a better understanding of the recovery process on micro- and macro-molecular level. Animal muscle injury models can roughly be categorized in mechanical-induced injuries, such as contusion injury, muscle overload, freeze injury and pressure injury, and chemical-induced injuries, e.g. by injecting myotoxic substances in skeletal muscle tissue, such as cardiotoxin (CTX), notexin, buvicain or glycerol(Mahdy 2018).

Upon muscle injury, the regeneration process exhibits distinct sequential phases, i.e. a destruction, repair and remodeling phase(Järvinen et al. 2005). The timing of each phase and of the up- and downregulation of particular pathways involved in the regeneration varies amongst mechanical- and chemical-induced muscle damage models(Lefaucheur and Bille 1995; Fink et al. 2003; Czerwinska et al. 2012), but also between different chemical-induced damage models(Mahdy et al. 2015). This has led to a welter of heterogeneous information on muscle regeneration, depending on the applied injury model, the type of injured muscle, the biochemical targets, parameters of muscle functionality, and the time point(s) within the regeneration process. Hence, it is not straightforward for researchers to predetermine and compare relevant outcome parameters with results from other studies, or to select an appropriate time point in the regeneration process for given outcome variables.

The present study sheds light on the complex multiplicity of information by describing both muscle histological changes and alterations in key molecular pathways at several time points during the regeneration process. Muscle injury was evoked by an acute CTX-injection. This model is highly eligible to study muscle recovery as the regeneration process occurs relatively efficient due to a proper blood supply and the preservation of basal lamina and microvasculature(Harris 2003; Czerwinska et al. 2012). CTX-injection is also less harmful for the animal compared to mechanical injuries(Couteaux et al. 1988). The CTX-induced regeneration model establishes similarities with diverse myopathies and might therefore serve as a tool to study these pathologies(Pessina et al. 2014; Sunitha et al. 2016). Besides, fundamental insights in muscle regeneration (both on molecular and histological level) contribute to our understanding of muscle injury and eventually to the development of novel strategies that might improve muscle healing upon injuries.

Recent advances in techniques, such as microarrays, allow to study a very broad range of targets following CTX injury(Goetsch et al. 2003; Yan et al. 2003). Despite the advantage of generating big data, this approach also encounters some limitations: i) mRNA expression is less physiologically relevant than posttranslational responses (e.g. phosphorylated proteins), ii) often small sample sizes (n=3/group), iii) hypothesis-free research which is not straightforward to interpret and to directly implement in physiological research. Therefore, we aim to study the (phosphorylated) protein levels of central targets that are expected to play a significant and distinct role in muscle regeneration and of which the changes during the regeneration process have not been described yet. We selected several stress markers (BiP, ERK1/2 and p38 MAPK and FOXO1/3a signaling) and anabolic markers (mTORC1 signaling pathway) which provide useful information about the response of the muscle tissue to injury, as well as the fiber type composition which is a key feature of the muscle phenotype.

Materials and Methods

Animal use – All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (KU Leuven Animal Ethics Committee; P168/2016). Forty-eight young (10 w), male C57BL/6 mice were purchased from Janvier Labs (France). In half of the group (n = 24), the middle portion of the m. *Tibialis Anterior* (TA) of both hindlimbs was injected with sterile CTX (10µM; L8102, Latoxan, France) dissolved in sterile 150µl phosphate-buffered saline (PBS), while the other half was injected with 150µl sterile PBS (n=24) with a BD microfine 300-µL U-100 insulin syringe. Prior to TA injection, mice were anaesthetized by intraperitoneal (IP) injection of $10\mu L \cdot g^{-1}$ body mass (BM) of saline solution containing 5% Rompun ($100mg \cdot mL^{-1}$ xylazine) and 10% Nimatek ($100mg \cdot mL^{-1}$ ketamine). In each condition, 8 mice were sacrificed respectively 2, 5 and 12 days following CTX or PBS injection. Mice abstained from food 2-3h prior to their sacrifice. TA muscles were surgically removed and weighed. One TA muscle was snap frozen in liquid nitrogen and stored at -80°C for later protein extraction, whereas the other TA muscle was frozen in liquid nitrogen-cooled isopentane and later sectioned at a thickness of 7 µm for histological analyses.

Histological analyses – Muscle tissue embedded in tissue freezing medium (Leica Biosystems, Germany) was frozen in liquid nitrogen-cooled isopentane. Serial 7-µm-thick cryosections were cut with a cryostat (Leica Biosystems CM1850, Germany) at −20°C. Prior to the histological analyses, cryosections were thawed at room temperature, washed with PBS and fixed with 4% paraformaldehyde. To permit qualitative analysis of morphological alternations, cryosections were stained with haematoxylin and eosin (H&E) (Sigma Aldrich, USA), and with fibronectin (FN) + Hoechst. For the FN staining, cryosections were blocked for 30min in PBS containing 5% bovine serum albumin (BSA). Next, cryosections were incubated for 1h at 37°C in a humid chamber with anti-FN antibody (Abcam ab2413; 1:200 dissolved in 1% BSA in PBS). After washing PBS, cryosections were incubated for 1h at room temperature with the conjugated donkey anti-rabbit IgG secondary antibody Alexa-488 (1:500), washed with PBS, incubated with Hoechst ($1\mu g \cdot mL^{-1}$) for 15min at room temperature. To obtain fiber type composition, cryosections were blocked for 2h in PBS containing 1% bovine serum albumin (BSA). Following permeabilisation in PBS (1% BSA+0.2% triton) for 15min, cryosections were incubated overnight at 4°C in a humid chamber with the following primary antibodies (Developmental Studies Hybridoma Bank, USA): BA-F8 (1:400, myosin heavy chain (MyHC) I), SC-71 (1:100, MyHC IIa), BF-F3 (1:300, MyHC IIb) and L9393 (1:500, Laminin, Sigma Aldrich, USA) dissolved in PBS. After washing in PBS, cryosections were incubated for 1h at room temperature with the following conjugated secondary antibodies (Life Technologies, USA): goat anti-mouse Alexa-488 IgG2 (1:300, MyHC I), goat anti-mouse Alexa-350 IgG1 (1:300, MyHc IIa), goat anti-mouse Alexa-594 IgM (1:300, MyHC IIb), donkey anti-rabbit Alexa-488 IgG (1:600, laminin). All immunofluorescence sections were mounted with Dako fluorescence mounting medium (Dako, S3023) and H&E-stained sections were covered with DPX mountant for histology (Sigma 06522).

Slides were visualized by fluorescence microscopy (Nikon E1000, Germany). The epifluorescence signal was recorded with FITC, DAPI and Texas Red excitation filters for visualization of MyHC I and cell membranes, MyHC IIa, and MyHC IIb, respectively, and with FITC and DAPI for visualization of FN and Hoechst, respectively. Muscle fibers of the full cryosection were classified as type I, type II, type IIb or unstained. Images of the slides were analyzed with ImageJ software (version 1.41, National Institutes of Health, USA).

Protein extraction – One total TA muscle was manually homogenized with a mortar, dissolved in ice-cold lysis buffer [1:10, w/v; 50mM Tris-HCl, pH 7.0; 270mM sucrose; 5mM EGTA; 1mM EDTA; 1mM sodium orthovanadate; 50mM glycerophosphate; 5mM sodium pyrophosphate; 50mM sodium fluoride; 1mM dithiothreitol; 0.1% Triton X-100; and a complete protease inhibitor tablet (Roche Applied Science, Belgium)] and centrifuged at 10 000g (25min, 4°C). Next, the supernatant was stored at -80°C. The protein concentration was assessed with the DC protein assay kit applying a BSA protein standard (Bio-Rad Laboratories, Belgium). Lysis buffer was added to equalize protein concentrations. Eventually, laemmli (20% of the total volume) was added to obtain muscle lysates.

Western blot analyses – The muscle lysate protein content (30-50µg) was separated using an SDS-PAGE (8-12% sodium acrylamide) and were transferred to polyvinylidene difluoride membranes, which were next blocked in tris-buffered saline tween-20 (TBS-T) with 5% BSA for 1h and incubated with the primary antibody, dissolved 1:200-1:10000 in 5% BSA in TBS-T, at 4°C overnight: phospho-Akt^{Ser473} (CST5171; 1:1000), phospho-mTOR^{Ser2448} (CST2971S; 1:1000), phospho-S6K1^{Thr389} (CST9206S; 1:1000), phospho-S6 Ribosomal Protein$^{Ser235/236}$ (CST2211; 1:1000), phospho-FoxO1^{Thr24}/FoxO3a^{Thr32} (CST9464S; 1:1000), phospho-p44/42 MAPK (Erk1/2)$^{Thr202/Tyr204}$ (CST9101; 1:1000), phospho-p38 MAPK$^{Thr180/Tyr182}$ (CST9215S; 1:1000), BiP (CST3177S; 1:1000) and GAPDH (CST2118S; 1:10000) (Cell Signaling Technology, The Netherlands). Secondary anti-mouse (1:7000) and anti-rabbit (1:5000) antibodies conjugated to horseradish peroxidase were applied to detect target proteins. Next, protein bands were quantified with the GeneSnap software (Syngene, UK). Since the CTX-injection increased the total protein form (Suppl. Fig. 1), proteins were presented relative to the total protein loading stained with Ponceau (Suppl. Fig. 2).

Statistical analyses – All values are presented as means±SEM. Non-normal data were log transformed and a two-way ANOVA with Tukey post-hoc tests was performed to assess differences among conditions and times. Statistical significance was accepted for $p<0.05$. All statistical analyses were performed with SPSS (Version 22.0.0.0; IBM Corp; USA).

Results

Muscle injury model (Table 1; Fig. 1; Fig. 2) – We analyzed the TA muscle 2, 5 and 12 days following PBS-injection to check whether the injection *per se* affected the selected molecular targets and muscle regeneration. PBS-injection did not induce skeletal muscle injury and regeneration (Fig. 1). Also, there was no effect of PBS-injection on BM or TA mass (table 1). CTX-injection induced no changes in BM compared to PBS, i.e. ΔBM (at pre-TA injection *vs.* at the day of sacrifice) was +0.05±0.4g in PBS at d2, and -0.8±0.1g at d2 ($p=0.38$), -0.5±0.4g at d5 ($p=0.69$) and +0.2±0.7g at d12 ($p=0.61$) in CTX (table 1). At d5, TA mass was significantly lower in CTX compared to PBS ($p<0.001$), while no

differences were observed at d2 (p=0.317) and d12 (p=0.937). CTX-injection effectively induced muscle injury and regeneration, as the destruction, repair and remodeling phase were clearly distinguishable at d2, d5 and d12. The early response upon CTX-induced muscle injury (i.e. d2) was characterized by infiltration of mononucleated cells (Fig.1 and Fig. 2) and interruption of the myofiber integrity (Fig.1). At the same time, FN, a glycoprotein present in the sarcolemma and connective tissue, was highly expressed in the damaged fibers, whereas FN was only expressed in the sarcolemma of PBS-injected fibers (Fig. 2). At d5, infiltrated cells remained present to a lesser extent and regenerating myofibers with centralized nuclei were present (Fig. 1). Furthermore, the FN expression was still present in some, but not all, fibers in damaged areas (Fig. 2). Infiltrated cells were present in the fibers that highly expressed FN, but not in undamaged fibers that only expressed FN in the sarcolemma (Fig. 2). Twelve days following CTX-injection, regenerating myofibers with centralized nuclei were enlarged compared to d5 (Fig. 1). The FN staining revealed that all fibers in regenerating areas expressed FN in the sarcolemma and not within the fibers (Fig. 2).

Shift in TA muscle fiber type (Fig. 3; Table 2) – As the amount of type I fibers was <1% and remained unchanged following CTX-injection, this fiber type was not included in the analyses. The **absolute amount** of type IIa fibers decreased at d2 compared to PBS (p=0.008), whereas unstained (type IIx and immature) fibers and type IIb fibers were not significantly different (p=0.30; p=0.20). At d5, the amount of type IIa fibers remained low *vs.* PBS (p=0.002) and the amount of unstained (p=0.004) and type IIb (p=0.001) were significantly higher than PBS. At d12, the amount of the type IIa, IIb and unstained fibers returned to PBS values. The **relative contribution** of each fiber type was assessed by dividing its respective absolute amount by the total amount of fibers. Upon CTX-injection, the type IIa fibers decreased ~11% at d2 *vs.* PBS (p=0.011), while unstained fibers were higher (+20%; p=0.008) and IIb fibers tended to be decreased (-10%; p=0.08). Five days upon CTX-injection, type IIa fibers remained low (-~12%; p<0.001), whereas type IIb and unstained fibers did not differ from PBS. At d12, none of the fiber types differed from PBS.

Fiber type specific characteristics (Table 2) – Two days upon CTX-injection, the minimum Feret diameter of all fiber types were non-significantly decreased compared to PBS. At d5, unstained fibers had a significantly lower minimum Feret diameter in CTX (21.9±1.3µm) *vs.* PBS (31.3±2.3µm; p=0.005). Accordingly, the diameter of type IIb fibers was lower five days following CTX-injection (25.2±1.9µm) compared to PBS (43.8±2.1µm; p<0.001). Twelve days following injection, there was a trend towards a decreased minimum Feret diameter of type IIa fibers in CTX (27.0±2.2µm) *vs.* PBS (33.5±4.2µm; p=0.075), and the diameter of unstained and type IIb fibers was significantly lower following CTX (28.4±3.4µm *vs.* 36.5±2.2µm; p=0.012 and 32.9±3.9µm *vs.* 47.6±4.6µm; p=0.003, respectively).

Muscle stress response (Fig. 4) – Stress markers and catabolic markers were severely affected by the CTX-injection. All markers, except for p-p38 MAPK, exhibited a significant treatment effect (p<0.05). First, BiP protein expression increased at d2 (22-fold *vs.* PBS; p<0.001) and gradually returned towards PBS-levels 5 days (2-fold *vs.* PBS; p=0.216) and 12 days following CTX-injection (p=0.788) (Fig. 4a). Second, the p-ERK1/2 MAPKs were significantly increased at d2 (~2-fold *vs.* PBS; p<0.05), peaked at d5 (~3-fold *vs.* PBS; p<0.001). The increment in p-ERK1/2 at d12 was less pronounced (1.7-fold *vs.* PBS; p=0.072 for p-ERK1 and p=0.163 for p-ERK2) (Fig. 4b, c). Phosho-p38 was increased at d2 (~2-fold *vs.* PBS; p=0.038) and decreased at d5 (0.4-fold *vs.* PBS; p=0.004), and was non-significantly decreased 12 days upon CTX-injection (0.5-fold *vs.* PBS; p=0.221) (Fig. 4d). Finally, p-FOXO1/3a expression was lower in the CTX-group at d2 (0.3-fold *vs.* PBS; p=0.002) and at d5 (0.4-fold *vs.* PBS; p=0.009), whereas the decreased expression at d12 was no longer significant (0.7-fold *vs.* PBS; p=0.302)(Fig. 4e).

Muscle anabolic response (Fig. 5) – All anabolic markers were significantly upregulated due to CTX compared to PBS injection (p<0.05). Phospho-Akt expression was increased 2 days (3-fold *vs.* PBS; p=0.02) and peaked 5 days following CTX-injection (4-fold *vs.* PBS; p<0.001), while the increase was no longer significant at d12 (2-fold; p=0.218) (Fig. 5a). In contrast to p-Akt, the increased p-mTOR expression 2 days upon CTX injection was not significantly different from PBS (1.4-fold *vs.* PBS; p=0.138). At d5, p-mTOR expression peaked (~3-fold *vs.* PBS; p<0.001) whereas at d12, there was no different expression compared to PBS (1-fold *vs.* PBS, p=0.969)(Fig. 5b). Phospho-S6K1 and p-S6rp were significantly increased at d2 (8- and 4-fold *vs.* PBS; p=0.004 and p=0.001, respectively) and at d5 (4-fold *vs.* PBS; p=0.089 and p<0.001, respectively) (Fig. 5c, d). At d12, both parameters did not significantly differ between PBS and CTX.

Discussion

This is the first study in which the posttranslational patterns of distinct key molecular pathways (mTORC1, MAPK, FOXO) and muscle histological changes are simultaneously displayed during muscle regeneration following CTX-induced injury. Two days upon injury, the stress response was characterized by an upregulation of BiP and a decreased amount of type IIa fibers. Next, at d5, unstained (immature and type IIx) and type IIb fibers were higher in CTX *vs.* PBS, with a concomitant

upregulation of mTORC1 and ERK1/2 signaling. Twelve days following the CTX-injection, the muscle phenotype was recovered towards the phenotype of a 'healthy' PBS-injected muscle.

Fiber type shift – We demonstrated that the slower type IIa fibers were more vulnerable to CTX-injection and that the formation of primarily fast muscle fibers preceded the slow fibers in the TA, i.e. slow type IIa fibers were decreased at d2 and d5 but not at d12 in CTX *vs.* PBS, whereas fast IIb fibers were higher at d5 compared to PBS (Fig. 3). These observations are in line with the findings of Launay et al. (2006), who studied the shift in fiber type following a CTX-injection in the m. *Soleus* and in the m. *Extensor digitorum longus* (EDL) of 10-wk old mice(Launay et al. 2006). The authors reported that the early response in the EDL was characterized by the presence of type IIx and type IIb fibers 5 days after injection, while type IIa fibers firstly appeared after 14 days. As the EDL is also characterized by a fast fiber phenotype predominance, it is not surprising that the fiber type shift in this muscle was very similarly to the shift in the TA. Accordingly, in the slow m. *Soleus*, type IIx and type IIb fibers were also the main fiber types five days following injection, while type IIa fibers were barely expressed. Seven days following CTX-injection, type IIa fibers became more apparent and at day 10, type I fibers appeared and their contribution increased until 28 days to become the mainly expressed fiber type. In agreement with our observations, these data confirm that in both 'slow' and 'fast' muscles, the formation of fast-twitch fibers precedes the slow-twitch fibers. However, in an 'intermediate' muscle, such as the m. *Gastrocnemius*, the formation of slow fibers preceded the fast fibers following a CTX-injection(Czerwinska et al. 2012). This discrepancy confirms that even within the same species and injury model, histological changes might profoundly differ amongst muscle types. Though, it cannot be excluded that different methodologies to assess the fiber type composition (immunofluorescence *vs.* MyHC gel electrophoresis) might also have contributed to the dissimilarities among these studies.
Besides the relative and absolute fiber type change during regeneration, we also related the regional appearances of specific fiber types to the muscle area integrity. At d2, the damaged muscle regions, characterized by mononucleated cell infiltration, were low in type IIa fibers, suggesting that these fibers are the most vulnerable to CTX-injection. At d5, it was apparent that muscle fibers with a centralized nucleus were almost exclusively type IIb and unstained fibers. Furthermore, the mimimum Feret diameter of type IIb and unstained fibers at d5 was significantly lower in CTX than in PBS, so it is likely that these fibers were newly formed between d2 and d5. One might suggest that unstained fibers at d5 represent immature embryogenic fibers. Indeed, classic work from Schiaffino showed the embryogenic and neonatal MHC isoforms are re-expressed after a denervation injury in rat skeletal muscle(Schiaffino et al. 1988). Also during recovery from a myotoxin injection in the rat m. *Soleus*, embryogenic and neonatal fibers appear very early (d3-d4) in the regeneration process(D'Albis et al. 1988; Jerkovic et al. 1997). Given their increased number and decreased size at d5, it is thus very likely that unstained fibers at d5 represent indeed immature embryogenic fibers, rather than fibers that lost their mature MyHC isoform due to the injury. At d12, besides a persistent loss of type IIa fibers (0.5-fold *vs.* PBS), the relative fiber type composition of CTX was largely recovered (resembling the PBS condition). This is in agreement with d'Albis et al., who concluded that two weeks upon CTX injury in rats, the muscle MHC pattern was very similar to controls(D'Albis et al. 1988). However, fiber sizes observed in our study remained small in PBS *vs.* CTX, indicating that a time frame >12 days is necessary for full fiber recovery towards a healthy muscle phenotype. To our knowledge, this is the first study that relates the fiber type shift to regional changes in the muscle cross-sectional area's integrity.

Muscle stress response - In their response upon injury, molecular targets exhibit differential expression patterns, reflecting their unique functioning during the regeneration process. CTX-injection in skeletal muscle tissue evokes numerous physiological changes, which might each (in)dependently activate stress responses. Both the absence and an exuberance of stress/inflammatory signaling upon injury negatively impact the structural and the functional recovery of skeletal muscle tissue(Bohnert et al. 2018; Yang and Hu 2018). This implies that a good understanding of the stress response is important for the development of strategies to improve recovery. When therapies for muscle regeneration are studied, changes in the expression of stress markers are often used to interpret the therapy-efficacy and to understand the underlying mechanism(s). Therefore, the selection of particular 'stress targets' is important, as their expression patterns strongly differ and might result in different interpretations.

BiP, a chaperone located in the lumen of the endoplasmic reticulum (ER), is a member of the heat shock proteins (HSPs). It binds newly-synthesized proteins but also marks proteins for the degradation via the ubiquitin-proteasome pathway, especially when unfolded proteins accumulate in the ER(Gething 1999). BiP is highly expressed in response to various physiological stresses, including acute muscle injuries(Senf 2013), and more specifically to changes in intracellular calcium concentrations, glucose starvation or heat(Waser et al. 1997). Since it is known that CTX-injection induces an influx of calcium in muscle tissue(Harvey et al. 1982; Ownby et al. 1993), it is likely that this contributed to the acutely elevated BiP expression at d2. In their microarray approach, Yan et al. observed that Hsp70 and Hsp86 expression levels were highly upregulated early upon CTX injury (d1-d3) and downregulated at a later phase (d14)(Yan et al. 2003). Accordingly, Senf et al. found that the Hspa1a (coding for HSP70) mRNA expression was significantly elevated 1 and 16 days following CTX-injection, while no increase was observed at day 4, indicating a biphasic response of HSP70 gene expression upon injury(Senf et al. 2013). It remains unclear to which purpose the second peak serves. Our data also revealed an acutely

elevated BiP protein expression at d2 and d5, but no second peak at d12. Interestingly, mitochondrial proteomic analysis revealed a downregulated expression of HSPd1 and HSPa9 (stress protein similar to Hsp70), with 3.3-fold and 2.5-fold, respectively, 3 days following a CTX injury(Ramadasan-Nair et al. 2014). However, one would expect that the expression of those proteins is increased to properly assemble unfolded polypeptides generated under stress conditions, i.e. tissue damage, in the mitochondrial matrix.

The BiP protein expression, which peaked at d2, preceded the upregulation of p-mTOR at d5. It is therefore more likely that the BiP levels reflect their function to chaperone damaged proteins for ubiquitine-proteasome mediated degradation(Selsby et al. 2007), rather than their function to chaperone newly formed proteins, since this would imply an earlier activation of mTORC1 signaling, the central regulator of protein synthesis. Accordingly, Kojima et al. suggested that an elevated HSP72 protein expression was unrelated to protein synthesis in the necrosis-regenerating process following CTX-injection(Kojima et al. 2007). Alternatively, increased BiP levels might also protect against muscle damage, e.g. through its antioxidant capacity(Yan et al. 2002), and thereby promoting muscle regeneration and recovery(McArdle et al. 2004; Miyabara et al. 2006; Selsby et al. 2007). In mice, genetically-induced HSP overexpression(McArdle et al. 2004) and heat-induced HSP upregulation(Selsby et al. 2007) were effective to attenuate the loss in muscle strength induced by eccentric contractions(McArdle et al. 2004) and to improve muscle reloading following immobilization-induced atrophy(Selsby et al. 2007) by a HSP-induced inhibition of the oxidative damage(Selsby et al. 2007).

The **MAPK** family is involved in directing the cellular response to diverse stress stimuli, including osmotic stress, mitogens, heat and proinflammatory cytokines(Roux and Blenis 2004). ERK1, ERK2 and p38 MAPK are engaged in both the adaptation to environmental stress, and in the regulation of cell proliferation, cell cycle progression, cell division and differentiation. Although they belong to the same family, the patterns of expression differed between p-ERK1/2 and p-p38 MAPK, suggesting a distinct role during muscle regeneration. The peak expression of p-ERK1/2 five days following injury was also observed in rats 5 days upon notexin injection in the m. *Soleus* (~+330% *vs.* intact muscle)(Richard-Bulteau et al. 2008). However, p-ERK1/2 was still increased 14 days following notexin injection although less pronounced (~+60%), whereas we observed no difference at d12 between CTX and PBS. Similar to our findings, p-p38 MAPK was not increased 5 days upon notexin injection, but at d14, notexin increased p-p38 MAPK expression *vs.* the intact control, while we observed a decreased expression 12 days following CTX injection. Differences in expression patterns of the MAPKs can be attributed to a different species (rat *vs.* mice), muscle (Soleus *vs.* TA) and injury model (notexin *vs.* CTX).
MAPK expression might be the result of the proinflammatory milieu in skeletal muscle upon CTX injury. However, if this were the case, MAPK expression would peak at d2, as mononuclear cell infiltration and muscle damage was more pronounced at this time point (Fig. 1), and not at d5. Alternatively, the higher expression might have been partly related to a shift in fiber type composition, i.e. due to the increase in IIb fibers. In C57BL/6 mice, p-ERK1/2 expression was shown to be ~2-fold greater in the fast TA and *Gastrocnemius* than in the slow *Soleus* muscle(Shi et al. 2007, 2008). P-p38 was not differentially expressed according to the muscle type(Shi et al. 2008). Since both ERK1/2 and p38 are involved in myoblast proliferation and differentiation(Jones et al. 2001; Keren et al. 2006; Li and Johnson 2006; Cho et al. 2007; Yokoyama et al. 2007; Segalés et al. 2016), it is hard to distinguish their distinct effects on the skeletal muscle regeneration process based on their expression.

FOXO transcription factors are involved in the muscle energy homeostasis and are key regulators of the protein breakdown through modulation of the ubiquitin-proteasome and autophagy-lysosomal pathways(Sanchez and Candau 2014). FOXO is deactivated when phosphorylated and translocates to the nucleus upon dephosphorylation to exert its function as a transcription factor and to activate atrogenes, such as MuRF-1 and MaFbx(Brocca et al. 2017). Therefore, the decreased phosphorylated protein expression might reflect the increased activation of protein breakdown pathways. This is in accordance with the increased BiP expression, which marks proteins for degradation. Upon muscle damage, protein breakdown, partly regulated via FOXO signaling, is necessary for the recycling of amino acids, which are used for tissue rebuilding(Bröer and Bröer 2017). At d12, phosphorylated FOXO is no longer significantly different between PBS and CTX, which indicates that catabolic processes are now decreased compared to the early phases in muscle regeneration.

Muscle anabolic signaling – Besides the upregulation of a muscle stress response, muscle regeneration is also characterized by an anabolic response(Ge et al. 2009). mTORC1 signaling, which is the key regulator of protein synthesis, plays a crucial role in tissue regeneration. Surprisingly, however, there are no data available on the expression pattern of this pathway during regeneration. Upstream of mTOR, Akt can be activated by stimuli such as insulin, growth factors, calcium and cAMP(Filippa et al. 1999). As mentioned above, CTX-induced muscle injury is characterized by the influx of calcium, which might have contributed to the upregulation of the Akt-mTOR axis. In the present study, the expression of the upstream mTORC1 intermediates, e.g. p-Akt and p-mTOR, peaked 5d following CTX injury compared to PBS. Accordingly, IGF-2 gene expression also peaked five days following CTX injury(Yan et al. 2003). It was earlier shown that mTORC1 signaling regulates the IGF-2 expression during skeletal muscle myogenesis, both in vitro(Erbay et al. 2003) and in vivo(Ge et al. 2009). It is remarkable that at d2, the downstream mediators p-S6K1 and p-rpS6 exhibited a relatively

higher protein expression (+740% and +260% *vs.* PBS) than the upstream proteins p-Akt and p-mTORC1 (+180% and +40% *vs.* PBS). It cannot be excluded that we might have missed an initial calcium-induced transient peak (before d2) in p-Akt and p-mTOR expression upon CTX-injection, leading to an upregulation of the downstream proteins observed at d5. However, it is also possible that the calcium might have directly activated S6K1, independent of Akt(Graves et al. 1997; Conus et al. 1998; Gulati et al. 2008).

It can be questioned whether changes in the expression of the markers of muscle anabolism, i.e. mTORC1 signaling, reflect the protein synthesis or are rather indicative of muscle remodeling. Only a few studies looked at the real protein dynamics in muscle injury models. When nerve transfection/denervation was applied as a muscle injury model in rats, the muscle protein breakdown was acutely upregulated, while the protein synthesis was elevated only 7-10 days following injury(Goldspink 1976, 1978; Goldspink et al. 1983). Lately, it was confirmed that muscle protein synthesis was elevated 14-28 days upon nerve constriction in rats, despite a loss of ~50% in muscle tissue (Langer et al. 2018). It should be noted, though, that the muscle atrophy process differs following a CTX-injection or following denervation. Still, these observations, which advocate that the mTORC1 activation reflects muscle remodeling rather than protein synthesis, correspond with our data as the upregulation of p-mTORC1 at d5 occurred with TA atrophy, i.e. a decreased muscle mass and a decreased IIb fiber size. It was therefore suggested that elevations in muscle protein synthesis, and thus in mTORC1 signaling, upon injury are indicative of muscle remodeling (e.g. myoblast proliferation, differentiation and fusion) rather than muscle growth *per se*(Ochala et al. 2011; Langer et al. 2018).

Integrative muscle signaling – Our data reveal that CTX severely impacts muscle histology and molecular signaling. To better understand the regeneration process, the potential physiological meaning(s) of the kinetics of each target should be interpreted in relation to the others. This might help researchers in their selection of targets and in data interpretation when applying a CTX injury model. In the present study, CTX activated two main pathways involved in the muscle regeneration, i.e. mTORC1 and ERK1/2 signaling. Both pathways exhibited similar expression patterns, i.e. a peak at d5 and a return towards baseline levels at d12. It was earlier suggested that the activation of mTORC1 signaling might be partially modulated by MAPK signaling(Roux and Blenis 2004; Miyazaki and Takemasa 2017). However, it is also possible that a same upstream mediator is at work for both targets. Given the elevated intracellular calcium concentrations upon muscle injury, it is possible that protein kinase C (PKC), induced by calcium, stimulated both mTORC1 signaling(Iijima et al. 2002; Fan et al. 2009; Miyazaki 2013; Osta et al. 2014) and ERK1/2(Cho et al. 2005; Tsao et al. 2013). In cardiac muscle cells, it was shown that mTORC1 activation was regulated through PKCs(Moschella et al. 2007). It remains to be established, whether this interaction also takes place in skeletal muscle cells. Furthermore, it should be noted that the activation of the analyzed stress markers and anabolic markers do not all necessarily result from the muscle fibers themselves, but might also be (partly) explained by immune and stromal cells, as their infiltration and proliferation takes places during the regeneration process. Indeed, the ERK pathway plays a central role in immune cells(Krzyzowska et al. 2010). Therefore, changes in immune cell number or phenotype during regeneration might also contribute to changes in pathway expression.

In conclusion, this is the first study that describes the molecular changes of pathways which play a central role during muscle regeneration following CTX-injection. Our study shows that the CTX-induced injury resulted in a rapid loss in type IIa muscle fibers, which was compensated by higher levels of type IIb fibers. Furthermore, CTX induced an early atrophic response, characterized by an acutely elevated increase in BiP and decrease in p-FoXO1/3a expression, eventually resulting in a decreased muscle mass and increase in type IIb muscle fibers. The activation of the mTOR and ERK1/2 pathway are very likely to be engaged in modulating cell proliferation and differentiation, and cell reconstruction via protein synthesis. The time-dependent and differential behavior of the targets indicate that researchers should carefully consider the selection of time point(s) upon injury and of specific targets to estimate the rate of injury/stress or recovery.

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Fig. 1 Regeneration phases of TA muscle fibers. Histological sections stained with H&E revealed muscle infiltration of mononucleated cells (red arrows) on day 2 (myolysis) and day 5 (early regeneration) following CTX-injection. At d5, damaged myofibers were replaced by small newly-formed myofibers with a centralized nucleus (white arrows). Twelve days upon CTX injury, myofibers with a centralized nucleus (white arrows) were enlarged and a larger contribution of myofibers with a nucleus in the periphery (green arrows) was observed. PBS-injection did not evoke muscle injury or regeneration, as healthy muscle fibers with peripheral nuclei were apparent 2, 5 and 12 days upon injection. Scale bar = 100 µm.

Fig. 2 Fibronectin (FN) in TA muscle during regeneration. Histological sections stained for FN and Hoechst (nuclei). Two days upon the cardiotoxin (CTX) injection, FN was highly expressed in damaged fibers, whereas FN was only expressed in the sarcolemma of PBS-injected fibers. At d5, the FN expression was still present in some but not all fibers in damaged areas. Infiltrated cells were present in the fibers that highly expressed FN (red arrows) but not in undamaged fibers that only expressed FN in the sarcolemma (white arrows). Twelve days following CTX-injection, the FN staining revealed that all fibers in regenerating areas expressed FN in the sarcolemma, but to a higher extent than the PBS-injected control. Scale bar = 100 µm.

Fig. 3 (n=7-8/condition): The amount of muscle type IIa (A), unstained (B) and type IIb fibers (C) during TA muscle regeneration expressed in absolute values. Data are expressed as mean ± SEM. * $p < 0.05$ *vs.* PBS. Representative muscle sections immunostained for myosin heavy chain (MyHC) I (green), MyHC IIa (blue), IIb (red) and laminin (green) (D). Scale bar = 100 µm.

Fig. 4 (n=7-8/condition): The expression of different proteins involved in the regulation of cellular stress in the TA following CTX injury. The target molecules are binding immunoglobulin protein (BiP), phospho (p)-Forkhead box O3 (FOXO), p-p38 mitogen-activated protein kinases (p38MAPK), p-extracellular signal-regulated kinases (ERK). Data are expressed in mean ± SEM. * $p < 0.05$ *vs.* PBS; † $p < 0.1$ *vs.* PBS.

Fig. 5 (n=7-8/condition): Expression of the mTOR signaling pathway in the TA following CTX injury. The target molecules are (from upstream to downstream) phospho (p)-Akt, p-mammalian target of rapamycin (mTOR), p-ribosomal protein S6 kinase beta-1 (S6K1) and p-ribosomal protein S6 (rpS6). Data are expressed in mean ± SEM. * $p < 0.05$ *vs.* PBS.

Body mass and m. *Tibialis Anterior* mass

	PBS			CTX		
	d2	**d5**	**d12**	**d2**	**d5**	**d12**
BM (start) (g)	25.0±0.6	25.8±0.7	26.7±0.8	25.0±0.2	25.8±0.5	26.5±0.5
BM (end) (g)	25.0±0.7	25.4±0.4	26.8±0.8	24.1±0.2	25.3±0.8	27.2±0.5
TA (mg)	51.2±1.3	50.6±1.0	49.9±1.0	49.1±1.8	41.1±0.9*****	50.0±1.9

Table 1 (n=7-8/condition). Data are presented as mean ± SEM. BM: body mass; TA: tibialis anterior; * P < 0.05 *vs.* PBS.

Relative muscle fiber type composition and minimum Feret diameter

		Day 2		Day 5		Day 12	
Fiber Type	Parameter	PBS	CTX	PBS	CTX	PBS	CTX
Type IIa	Frequency (%)	16.0±3.1	4.7±1.5 *****	17.2±2.7	4.7±2.1 *****	19.4±4.2	10.6±2.4
	Min Feret diam (µm)	33.5±1.3	28.3±2.1	27.4±2.9	29.8±1.3	33.5±4.2	27.0±2.2 †
Type IIx	Frequency (%)	32.0±5.6	54.1±7.9 *****	28.6±2.7	39.0±4.1	24.5±5.2	31.4±5.0
	Min Feret diam (µm)	36.0±1.3	33.6±1.7	31.3±2.3	21.9±1.3 *	36.5±2.2	28.4±3.4 *
Type IIb	Frequency (%)	52.0±4.1	41.2±6.8 †	54.2±1.8	56.2±2.6	56.1±4.0	57.9±3.9
	Min Feret diam (µm)	47.0±0.8	42.8±2.8	43.8±2.1	25.2±1.9 *	47.6±4.6	32.9±3.9 *

Table 2 (n=6-8/condition). Data are presented as mean ± SEM (n=8). Min Feret diam: minimum Feret diameter; * p < 0.05 *vs.* PBS; † p between 0.05-0.1 *vs.* PBS.