Ibuprofen does not impair skeletal muscle regeneration upon cardiotoxin-induced injury

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Short title: Ibuprofen & muscle regeneration upon cardiotoxin injury

Summary: Muscle regeneration is regulated through interaction between muscle and immune cells. Studies showed that treatment with supra-physiological doses of Non-Steroidal Anti-Inflammatory Drug (NSAID) abolished inflammatory signaling and impaired muscle recovery. The present study examines the effects of pharmacologically-relevant NSAID treatment on muscle regeneration. C57BL/6 mice were injected in the tibialis anterior (TA) with either PBS or cardiotoxin (CTX). CTX-injected mice received ibuprofen (CTX-IBU) or were untreated (CTX-PLAC). After 2 days, *Il-1β* and *Il-6* expression was upregulated in the TA of CTX-IBU and CTX-PL *vs.* PBS. However, *Cox-2* expression and macrophage infiltration were higher in CTX-PL *vs.* PBS, but not in CTX-IBU. At the same time, anabolic markers were higher in CTX-IBU *vs.* PBS, but not in CTX-PL. Nevertheless, ibuprofen did not affect muscle mass or muscle fiber regeneration. In conclusion, mild ibuprofen doses did not worsen muscle regeneration. There were even signs of a transient improvement in anabolic signaling and attenuation of inflammatory signaling.

Key words: NSAID; inflammation; mTORC1 signaling; cyclooxygenase; muscle recovery

INTRODUCTION

Skeletal muscle injuries frequently occur, e.g. due to work or traffic accidents or sport traumas. If the muscle tissue is incompletely or improperly regenerated, muscle capacity can be permanently impaired, which might limit daily-living activities. The muscle recovery process is closely regulated by inflammatory signaling, e.g. cytokines produced by neutrophils and macrophages, which modulate other immune cells, myocytes and their interaction. The cytokines and mitogens that are released in the injured skeletal muscle tissue promote the synthesis of prostaglandins (PGs) in inflammatory cells. Besides their modulatory role in inflammation, PGs are involved in muscle metabolism, and more specifically in myoblast proliferation, differentiation and fusion(Leng and Jiang, 2019; Mo et al., 2015), and in the regulation of muscle protein synthesis(Weinheimer et al., 2007) and degradation(Markworth and Cameron-smith, 2011; Rodemann et al., 1982; Vandenburgh et al., 1990). Therefore, PGs have gained much interest as mediators of the skeletal muscle regeneration process.

There are many therapies available to modulate muscle regeneration, including RICE (rest, ice, compression and elevation), physical therapy and medication. Especially, non-steroidal anti-inflammatory drugs (NSAIDs) are very commonly used for muscle regeneration purposes(Elnachef et al., 2008). Through COX-inhibition, NSAIDs attenuate the PG production, and thereby dampen inflammatory signaling(Green, 2001). Although very popular, NSAIDs play a dual role in muscle regeneration. Shortly upon injury, NSAIDs seem to induce a more complete functional recovery. Mishra et al. (1995) observed faster muscle strength regeneration in rabbits when an exercise-induced muscle injury was treated with flurbiprofen(Mishra et al., 1995). However, this effect was only temporary (~7 days). On day 28, NSAID treatment resulted in a deficit in muscle strength compared to the untreated group(Mishra et al., 1995). This indicates that the effect of NSAID treatment on skeletal muscle tissue is not as straightforward as often suggested. Indeed, NSAIDs might improve the performance upon injury, through inhibition of the initial inflammatory reaction and concomitant pain.

Yet, the role of NSAID-induced inhibition of the inflammatory response in muscle regeneration remains controversial(Järvinen et al., 2013; Mishra et al., 1995; Prisk and Huard, 2003). Two rodent studies raised caution about the use of COX inhibitors in the context of muscle recovery, as they showed that COX inhibition impaired the skeletal muscle regeneration process, mainly via a dysregulated inflammatory signaling(Bondesen et al., 2004; Novak et al., 2009). However, in both studies, COX signaling was inhibited to a supra-physiological extent, either pharmacologically or via COX knockout. However, a minimal inflammatory response by immune cells (i.e. phagocytosis of cellular debris and the release of chemoattractants and growth factors) facilitates myogenesis during regeneration(Chazaud et al., 2003; Tidball, 2005). Therefore, we hypothesize that a mild, pharmacologically-relevant COX inhibition, that only slightly attenuates the inflammatory response, does not impair muscle regeneration. The present study examines whether mild NSAID treatment affects the skeletal muscle regeneration process upon cardiotoxin-induced muscle injury, through changes in muscle histology, muscle anabolic signaling (mTORC1) and muscle inflammatory signaling in C57BL/6 mice.

METHODS

Animal use – The experiment was approved by the KU Leuven Animal Ethics Committee (P168/2016), and all methods were performed in accordance with the relevant guidelines and regulations. Seventy-two young (10 w) male C57BL/6 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice were supplemented with either ibuprofen ($20 mg \cdot kg^{-1} \cdot d^{-1}$), dissolved in liquid diet or liquid diet (F1259SP, Bio-Serve; Flemington, USA) as such. To allow appropriate ibuprofen treatment, the liquid diet of individually housed mice was daily weighed and ibuprofen doses in the liquid diet were adapted to achieve $20 mg \cdot kg^{-1} \cdot d^{-1}$. Three days following the start of the supplementation, the m. *Tibialis Anterior* (TA) of both hindlimbs of the ibuprofen-treated group (n=24; CTX-IBU) and of half of the

placebo group (n=24; CTX-PL) was injected with CTX (10µM in 150µl phosphate buffered saline (PBS); L8102, Latoxan, Portes-lès-Valence, France), while the other half of the placebo group was injected with PBS (control group) (n=24). Prior to the injection, mice were anaesthetized by intraperitoneal injection of 10µl·g^{-1} BM of saline solution containing xylazine (100mg·mL^{-1}, Rompun 5%) and ketamine (100mg·mL^{-1}, Nimatek 10%). In each condition, 8 mice were sacrificed respectively 2, 5 and 12 days following CTX- or PBS-injection. TA muscles were surgically removed, weighed and snap frozen in liquid nitrogen and stored at -80 °C for biochemical and histological analyses. A schematic overview of the study design is presented in Fig. 1.

Histological analyses – Muscle tissues were embedded in tissue freezing medium (Leica Biosystems, Wetzlar, Germany) and frozen in liquid nitrogen-cooled isopentane. Serial cryosections (7µm thick) were cut with a cryostat (Leica Biosystems CM1850, Wetzlar, Germany) at −20°C. Prior to histological analyses, cryosections were thawed at room temperature (RT), washed with PBS and fixed with 4% paraformaldehyde. To permit qualitative analysis of muscle fiber morphological alternations, cryosections were stained with haematoxylin and eosin (H&E) (Sigma Aldrich, St Louis, USA). In addition, to assess muscle regeneration, the characteristics of myofibers with a centralized nucleus were measured in the entire muscle section of mice in CTX-PL and CTX-IBU at d5, as this time point corresponds with maximal regeneration(Chen et al., 2005; Garry et al., 2016). As expected, no fibers with a centralized nucleus were observed in PBS. For the muscle fiber type composition and F4/80-positive (F4/80^{+}) macrophage staining, cryosections were blocked for 2h in PBS containing 1% bovine serum albumin (BSA). Following permeabilisation in PBS (1% BSA + 0.2% triton x-100) for 15min, cryosections were incubated overnight at 4°C in a humid room with primary antibodies; for fiber typing (Developmental Studies Hybridoma Bank, Iowa, 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, St Louis, USA); for macrophage staining (Cell Signaling Technology, Leiden, The Netherlands): F4/80 (1:250; D2S9R) dissolved in PBS. After washing in PBS, cryosections were incubated for 1h at RT with the conjugated secondary antibodies; for fiber typing (Life Technologies, California, 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); for macrophage staining: donkey anti-rabbit Alexa-488 IgG (1:300, F4/80).

Slides were visualized by fluorescence microscopy (Nikon E1000, Nikon, Boerhavedorp, Germany). The epifluorescence signal was recorded with FITC (MyHC I, cell membranes and F4/80^{+} macrophages), DAPI (MyHC IIa) and Texas Red (MyHC IIb) excitation filters. Muscle fibers of the entire muscle section were classified as type I, type IIa, type IIb or immature + type IIx (unstained fibers). The F4/80^{+} macrophages infiltration was quantified by the mean colour density of the whole muscle cryosection. Muscle cryosections were analyzed with ImageJ software (version 1.41, National Institutes of Health, USA) by investigators who were blinded to the experimental conditions.

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

Western blot analyses – The lysate protein content (30-50µg) was separated using an SDS-PAGE gel (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), phospho-mTOR (Ser2448; CST2971S), phospho-S6 Kinase (Thr389; CST9206S), GAPDH (CST2118S) (Cell Signaling Technology, Leiden, The Netherlands). Secondary anti-mouse (1:7000) and anti-rabbit (1:5000) antibodies conjugated to horseradish peroxidase were applied to detect target proteins. Target protein bands were quantified with the GeneSnap software and tools (Syngene, Cambridge, UK). Since CTX-injection increased the total protein form of mTOR and S6K1 compared to PBS, proteins were presented relative to GAPDH.

RNA extraction and real-time quantitative PCR – Total RNA was extracted from ~20mg of muscle tissue, homogenized in 1ml Trizol reagent (Thermo Fisher Scientific, Waltham, USA). The RNA purity and concentration were assessed by Nanodrop (Thermo Fisher Scientific) spectrophotometry. Reverse transcription was performed from 1µg RNA using the iScriptcDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, USA), following the manufacturer's instructions. The PCR was run using the following conditions: 2min at 95°C, followed by 50 cycles of 15s at 95°C, 30s at 60°C and 40s at 72°C. Each reaction was processed in a 25µL volume containing 12.5µL Promega PCR Master Mix, 1µL of each primer (0.1µM), 8.5µL RNAse free H2O and 2µL cDNA (25ng). Mouse-specific primers for *Cyclooxygenase-2* (*Cox-2), Interleukine (Il)-6* and *Il-1*β were designed (Table 1). To compensate for variations in input RNA amounts and efficiency of reverse transcription, ribosomal protein L41 (rpl41) mRNA was quantified, and results were normalized to those values using the $\Delta\Delta C_T$ method. The results are expressed in arbitrary units.

Statistical analyses – All values are presented as average ± SEM. A two-way ANOVA across conditions and time was performed. Post-hoc analyses were Tukey-corrected and statistical significance was accepted for $p < 0.05$. All statistical analyses were completed with SPSS (Version 22.0.0.0; IBM Corp, NY, USA).

RESULTS

Muscle injury and regeneration – CTX-injection effectively induced muscle injury and regeneration, as the destruction, repair and remodeling phase were clearly distinguishable at 2 (d2), 5 (d5) and 12 (d12) days following the injection, both in untreated (CTX-PL) and ibuprofen-treated (CTX-IBU) group (Fig. 2). The early response upon CTX-induced skeletal muscle injury (d2) was characterized by inflammatory cell infiltration and interruption of the myofibers' integrity. At d5, inflammatory cells remained present and new, small regenerating myofibers with centralized nuclei appeared. The relative contribution of fibers with a centralized nucleus (% to the total fiber number) did not differ between CTX-PL (42.6±6.4%) and CTX-IBU (36.5±3.2%; p=0.44), neither did the mean cross sectional area (667±34 µm² in CTX-PL and 663±45 µm² in CTX-IBU; p=0.94) and perimeter (99±3µm in CTX-PL and 98±3 µm in CTX-IBU; p=0.83) of fibers with a centralized nucleus. Twelve days following CTX-injection, myofiber integrity was partially restored and regenerating myofibers with centralized nuclei were enlarged compared to d5.

Muscle mass – The mass of the non-injected hind limb muscles (gastrocnemius, soleus, quadriceps) was unaffected by condition or time. TA muscle mass was not different between conditions at d2 and d12, while at d5, TA mass was significantly lower in CTX-injected conditions compared to the PBS-injected controls (CTX-PL: 40.1±0.9 mg; CTX-IBU: 40.1±2.4 mg; PBS: 50.6±1.0 mg; $p < 0.001$). There was no difference in TA muscle mass between CTX-PL and CTX-IBU at the different time points.

Muscle fiber composition (Table 2; Fig. 3) – CTX-injection induced a shift in muscle fiber type composition. Two days following CTX-injection, there was a ~10% loss of type IIa muscle fibers compared to PBS, irrespective of treatment (CTX-PL: p=0.006; CTX-IBU: p=0.032; Fig. 3A), while unstained muscle fibers (immature + type IIx) increased upon CTX-injection (+30% in CTX-PL, p<0.001; +20% in CTX-IBU, p=0.02; Fig. 3C). Type IIb muscle fibers decreased in CTX-PL (-17.1% *vs.* PBS; p=0.025), but not in CTX-IBU (p=0.398; Fig. 3B). Five days upon CTX-injection, type IIa

muscle fibers remained low in CTX-PL (-12.5%; p=0.007) and CTX-IBU (-10.0%; p=0.041; Fig. 3A) *vs.* PBS, while unstained fibers (Fig. 3C) and type IIb muscle fibers (Fig. 3B) did not differ between conditions. At d12, type IIa muscle fibers tended to be lower in CTX-PL compared to PBS (-17%; p=0.10), whereas this was not the case for CTX-IBU (-10%; Fig. 3A). In line with d5, unstained and type IIb fibers were similar between conditions (Fig. 3B,C). At none of the time points and for none of the fiber types, there were significant differences between the CTX-PL and CTX-IBU condition (p>0.05).

F4/80$^+$ macrophage infiltration (Fig. 4) – Macrophage infiltration was increased at d2 both in CTX-PL and in CTX-IBU. However, the increase compared to PBS only reached significance in CTX-PL (p=0.029), while there was no significant difference between CTX-IBU and PBS (p=0.186). At d5, CTX-injection increased macrophage infiltration, irrespective of treatment. At d12, macrophage infiltration in the CTX-injected conditions was comparable to PBS.

Muscle inflammation (Fig. 5) – Upon CTX-injection, inflammatory signaling was increased compared to the PBS-injection. *Cox-2* (Fig. 5A) mRNA expression at d2 was more elevated in CTX-PL (19.5±12.2 a.u.) *vs.* PBS (1.0±0.1 a.u.; p=0.017), while this was not the case for CTX-IBU (12.0±3.0 a.u.; p=0.251). However, there was no significant difference between CTX-PL and CTX-IBU (p=0.68). At d5 and d12, *Cox*-2 mRNA expression did not differ between the three conditions. Both *Il-6* (Fig. 5B) and *Il-1β* (Fig. 5C) mRNA expression were elevated 2 days following CTX-injection, irrespective of treatment. Similarly to *Cox-2*, their expression was not different from PBS at d5 and d12.

Muscle anabolism (Fig. 6) – Members of the mTORC1 signaling pathway were upregulated 2 and 5 days following CTX-injection compared to PBS. At d2, the upstream mediator p-Akt (Fig. 6A) was higher in CTX-IBU (0.47±0.12 a.u.) and in CTX-PL (0.31±0.05 a.u.) compared to PBS (0.10±0.02 a.u.). However, significance was only reached in CTX-IBU (p=0.022 *vs.* PBS) and not in CTX-PL (p=0.353 *vs.* PBS). Similarly, compared to PBS (0.29±0.02 a.u.), p-mTOR (Fig. 6B) expression was higher in CTX-IBU (0.69±0.09 a.u.; p=0.047), but not in CTX-PL (0.49±0.04 a.u.; p=0.708) at d2. Still, p-mTOR expression did not significantly differ between CTX-PL and CTX-IBU (p=0.64). Downstream of mTOR, p-S6K1 (Fig. 6C) was significantly upregulated 2 days following CTX-injection compared to PBS (0.06±0.02 a.u.), irrespective of treatment (CTX-PL: 0.58±0.18 a.u.; p=0.037; CTX-IBU: 0.70±0.18 a.u.; p=0.007). Similarly, five days following CTX-injection, p-Akt expression was significantly increased compared to PBS (0.29±0.10 a.u.), irrespective of treatment (CTX-PL: 1.24±0.10 a.u.; p<0.001; CTX-IBU: 1.10±0.19 a.u.; p<0.001). A similar pattern was observed for p-mTOR expression, i.e. significantly higher values in CTX-PL (1.15±0.13 a.u.; p<0.001) and in CTX-IBU (1.12±0.24 a.u.; p<0.001) compared to PBS (0.36±0.07 a.u.). Phospho-S6K1 was more elevated in CTX-PL (0.75±0.21 a.u.; p=0.008) and tended to be higher in CTX-IBU (0.60±0.23 a.u.; p=0.066) compared to PBS (0.12±0.05 a.u.). However, there was no difference between CTX-PL and CTX-IBU (p=1.0). Twelve days after the CTX-injection, the phosphorylation state of members of the mTORC1 pathway were similar to PBS.

DISCUSSION

In the present study, we examined whether a mild dose of ibuprofen impaired muscle regeneration upon injury and whether markers of inflammatory and anabolic signaling were affected. CTX upregulated muscle inflammation in both CTX-PL and CTX-IBU. A mild ibuprofen treatment did not strongly affect the CTX-induced inflammatory signaling. However, whereas *Cox-2* expression and F4/80$^+$ macrophage infiltration were significantly higher in CTX-PL than in PBS, both parameters were not significantly higher in CTX-IBU vs. PBS. This suggests that mild NSAID treatment upon muscle injury slightly attenuates the CTX-induced muscle inflammatory milieu. Furthermore, ibuprofen also stimulated the mTORC1 pathway. Eventually, none of these ibuprofen-induced changes in inflammatory or anabolic

signaling affected muscle regeneration, as the amount and size of regenerating fibers following CTX-injection did not differ between CTX-IBU and CTX-PL. It should be noted that IBU/PL treatment started 3 days before the TA injection. Although it is unlikely that IBU treatment before injury interferes with molecular signaling in the muscle tissue, this does not parallel real life situations in which treatments are implemented after the trauma.

CTX injection severely affected the fiber type composition, i.e. a loss in type IIa muscle fibers at d2, d5 and d12. Furthermore, at d2, CTX induced an increase in unstained fibers and a decrease in type IIb muscle fibers. Unstained fibers refer to fibers that express either immature myosin isoforms or the type IIx myosin isoform. Given the increased contribution of unstained fibers very early after injury, it is very likely that this can be attributed to a sudden increase of fibers expressing embryonic and neonatal myosin heavy chains, rather than type IIx fibers(Jerkovic et al., 1997). The loss of type IIb fibers at d2 and of type IIa fibers at d12 was partly counteracted by the ibuprofen treatment. This resulted in a muscle phenotype in CTX-IBU that is more similar to the healthy PBS phenotype compared to the untreated injury group. This is the first study that reports a protective effect of ibuprofen on the shift in muscle fiber type following acute injury. One earlier study reported that ibuprofen induced a fiber type shift in rats, i.e 2 weeks of ibuprofen treatment decreased type IIb and increased IIx muscle fibers during normal cage activity, while type IIa muscle fibers were decreased after a 2-week running protocol (5x per week)(Rooney et al., 2016). This confirms that ibuprofen might indeed interfere with the biomolecular mechanisms which underlie a shift in muscle fiber type. However, future studies should further reveal how ibuprofen is involved in the regulation of the fiber type composition and whether this ibuprofen-induced shift is beneficial for muscle recovery and/or functionality.

Muscle traumas, i.e. due to fractures(Hurtgen et al., 2016), strain injuries(Bayer et al., 2018) or excessive exercise(Rubio-Arias et al., 2018), are accompanied by an inflammatory response, which plays a key role in the regeneration process. On the one hand, muscle recovery can be impaired due to diminished inflammation, i.e. through macrophage depletion(Liu et al., 2017; Xiao et al., 2016) or via pharmacological interventions(Bondesen et al., 2004). On the other hand, excessive inflammation, i.e. neutrophil-derived free radicals, might stimulate the skeletal muscle damage(Toumi and Best, 2003). Therefore, a tight regulation of inflammatory signaling following injury is of importance to maximize muscle recovery. Lately, much evidence indicates that NSAID use might impair muscle regeneration upon injury(Trappe et al., 2002; Xian and Zhou, 2009), but also the increase in muscle mass, muscle strength(Lilja et al., 2017) and protein synthesis following resistance exercise(Trappe et al., 2002). Therefore, it is advised to discontinue prophylactic use of NSAIDs and restrict its use to the minimal dose and duration for the short-term management of acute pain and inflammation(Warden, 2010). The data of the present study suggest that moderate ibuprofen treatment does not impair tissue regeneration. However, it should be noted that the upregulated inflammatory signaling and injury features following CTX-injection(Dalle et al., 2020) are way beyond the inflammatory response upon muscle trauma, e.g. due to sport injury.

It was earlier established that inflammatory signaling negatively impacts muscle metabolism in different contexts such as sepsis(Hasselgren et al., 2005), sarcopenia(Dalle et al., 2017) and injury(Toumi et al., 2006). Therefore, we studied whether suppression of inflammatory signaling affected the markers of muscle anabolic signaling, i.e. the mTORC1 signaling pathway. Our findings suggest that mild ibuprofen-induced selective downregulation of muscle inflammation does not impair mTORC1 signaling upon injury and might even upregulate anabolism during early muscle regeneration. Similarly, in a murine muscle overload model, COX-2 inhibition did not affect mTORC1 signaling(Novak et al., 2009). This might be explained by the 'overloaded' muscle model which is physiologically different from muscle injury. Interestingly, Markworth et al. (2014) observed in healthy young subjects that ibuprofen acutely ingested prior to (-30min; 400mg) and following (+6 and +12h; 2 x 400mg) a resistance exercise bout increased p-S6K1^{Thr387} expression 24h post-exercise, however, without

upregulation of p-Akt^{Ser473}(Markworth et al., 2014). In arthritic rats, pharmacological COX-2 inhibition increased the expression of IGF-1(Granado et al., 2007). Unfortunately, the authors did not look at mTORC1 signaling, which occurs downstream of IGF-1. Also in other cell types, COX-2 signaling was shown to interact with mTORC1 signaling. Treatment of pancreatic β cells with PGE_2, a downstream effector of COX-2, downregulated p-Akt at Ser473 and Thr308(Meng et al., 2006). Despite varying contexts, ranging from exercise-induced muscle damage in humans to CTX-induced muscle injury in mice, these data suggest that there might be a modulatory effect of ibuprofen on muscle anabolic signaling, probably at least partly regulated via COX-2 signaling. Unfortunately, despite its potential role in skeletal muscle protein synthesis(Rodemann and Goldberg, 1982), the association between COX-2 and the mTORC1 signaling pathway is barely studied in skeletal muscle tissue. Therefore, it remains to be determined whether modulations of COX signaling in the present study contributed to a stimulation in mTORC1 signaling early upon CTX-injection.

Most evidence indicates that COX-2 inhibition upon muscle injury negatively affects recovery in mice. Similarly to our findings, pharmacological COX-2 inhibition decreased the inflammatory cell infiltration in a freeze-induced muscle injury(Bondesen et al., 2004) and in a muscle overload model(Novak et al., 2009). Contrarily to our findings, this resulted in impaired muscle regeneration, i.e. decreased myofiber size(Bondesen et al., 2004) or muscle mass(Novak et al., 2009). In addition, *in vitro* evidence indicated that COX-2 inhibition blunted myogenesis in C_2C_{12} muscle cells. Therefore, it is generally stated that COX-2 inhibition upon muscle injury is very likely to impair muscle recovery. This might be due to PG-dependent inflammatory effects(Prisk and Huard, 2003). Furthermore, a downregulation in the PG-induced stimulation of satellite cells(Bondesen et al., 2004; Mendias et al., 2004; Mikkelsen et al., 2009) due to NSAID use might also contribute to the decreased muscle regeneration and muscle strength after muscle repair(Mackey et al., 2012).

In the present study, there was no detrimental effect of NSAID use on the skeletal muscle regeneration. The discrepancy between our data and the studies mentioned above might be explained by the use of a different muscle injury/regeneration model and/or differences in COX-inhibition. However, the degree of COX-2 inhibition also varied between the studies, due to different doses and the use of different NSAIDs. Despite a dose of 20 $mg \cdot kg^{-1}$ BM ibuprofen in our study, *Cox-2* mRNA expression was only modestly attenuated (~40%). Substantial lower doses of 6 $mg \cdot kg^{-1}$ BM SC-236 and 10 $mg \cdot kg^{-1}$ BM of NS-398 were used in the study of Bondesen et al. and Novak et al., respectively. Unfortunately, in these studies, *Cox-2* mRNA or protein expression following injury was not compared between an untreated group (e.g. CTX-PL) and a group in which COX-2 was inhibited. Still, it is very likely that their pharmacological COX-2 inhibition was far more potent since the IC_{50} values for COX-2 activity, though not assessed in skeletal muscle cells, were much lower compared to that of ibuprofen(Bhardwaj et al., 2014; Kato et al., 2001; Kaur et al., 2018). It would be useful if future studies confirm the downregulation of COX-2 following NSAID treatment to ensure that the modifications in muscle regeneration can be mechanistically ascribed to COX-2.

Generally, findings obtained in studies that use very potent COX-2 inhibitors(Bondesen et al., 2004; Markworth and Cameron-Smith, 2013; Mendias et al., 2004; Novak et al., 2009) should be interpreted with caution when conclusions are translated to a human setting. Upon injury, people are very likely to use a moderate COX-inhibitor such as ibuprofen, rather than very potent COX-2 inhibitors such as SC-236 and NS-398. Whereas ibuprofen partly inhibits COX-2 and concomitantly attenuates inflammation, it still allows sufficient COX-2 signalling to enable appropriate muscle regeneration. Contrarily, very potent inhibitors block COX-2 signalling to a much higher extent, which does not allow inflammatory processes that are necessary for appropriate myogenesis, e.g. phagocytosis of debris and secretion of growth factors by macrophages, and might therefore impair muscle recovery. Accordingly, when 6-week resistance exercise was combined with chronic naproxen sodium (IC_{50} COX-2(Noreen et al., 1998) and PGE_2(Gierse et al., 1999) similar to ibuprofen) treatment in healthy males, COX-PG signalling was

downregulated(Brewer et al., 2015). Still, this did not affect the muscular adaptations to resistance exercise, confirming that mild, 'pharmacologically-relevant' COX inhibition does not necessarily impair muscular adaptations. Interestingly, in an elderly population, 12 weeks of resistance training combined with chronic ibuprofen supplementation ($1.2g.d^{-1}$) resulted in larger gains in muscle mass and muscle strength compared to an untreated group(Trappe et al., 2011). Future research should further reveal how COX-2 inhibitors with different potencies regulate the inflammation and regeneration upon muscle stress.

NSAID use is very common for the treatment of inflammation and pain, e.g. due to muscle injury. However, different studies raised caution about the use of NSAIDs for muscle recovery purposes, as they impaired regeneration(Bondesen et al., 2004; Mishra et al., 1995; Novak et al., 2009). These studies used strong COX inhibitors to totally abolish muscle inflammatory signaling, and concluded that NSAID use impairs muscle regeneration(Almekinders and Gilbert, 1986; Bondesen et al., 2006; Trappe et al., 2002). Although observed in experimental injury models, this might (unjustifiably) discourage muscle injury patients to use this treatment for recovery purposes. In the present study, a moderate COX inhibition through ibuprofen treatment did not oppose muscle regeneration. On the contrary, this treatment seemed to attenuate early inflammatory signaling, i.e. C*ox-2* expression and $F4/80^{+}$ macrophage infiltration, and to transiently upregulate markers of muscle anabolism such as p-Akt and p-mTOR. These data suggest that, in contrast to supra-physiologically strong COX inhibitors, there are no reasons to believe that a mild and pharmacologically-relevant ibuprofen treatment impairs the muscle recovery process.

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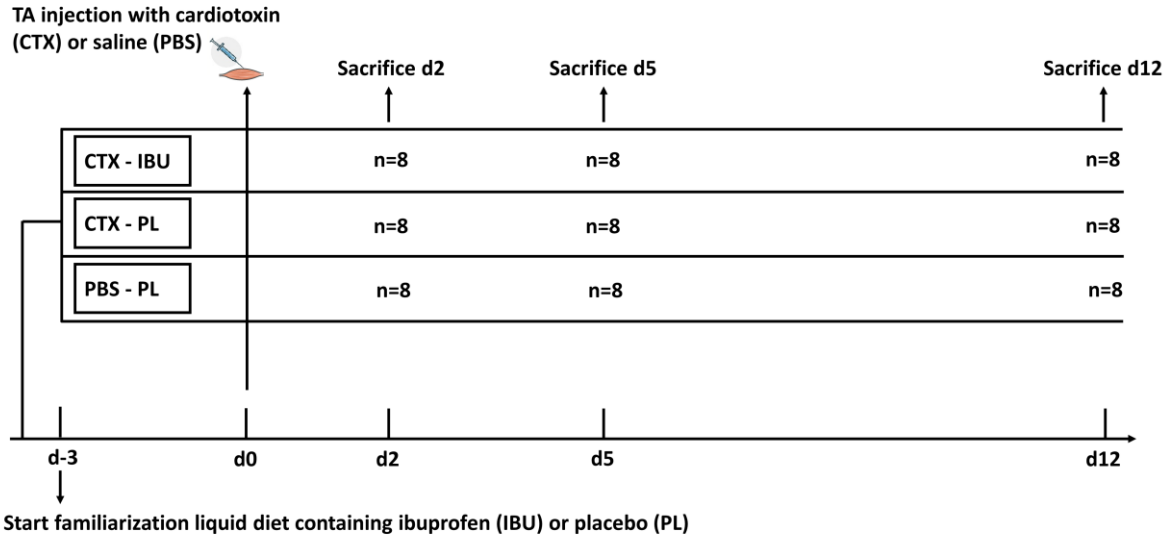

Fig. 1: Schematic overview of the study protocol.

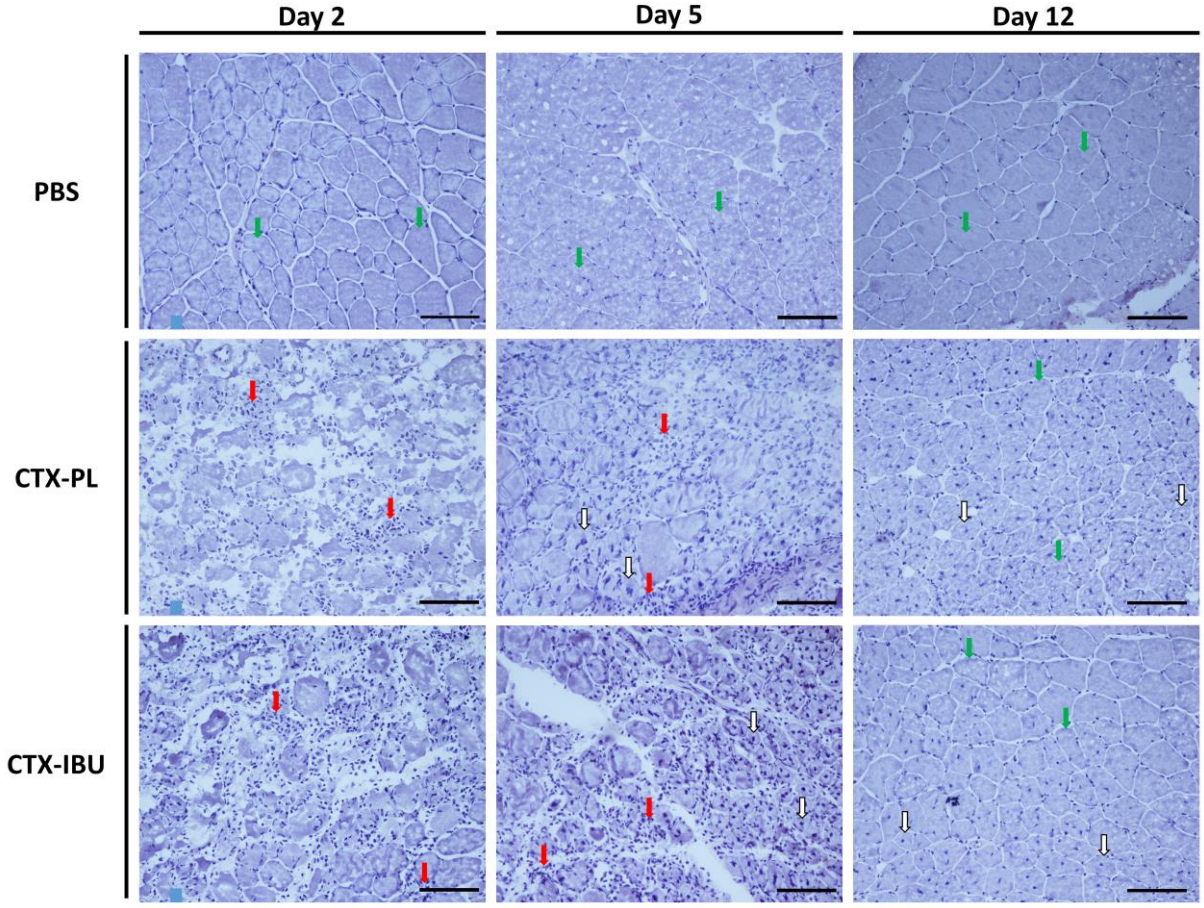

Fig. 2: Regeneration phases of TA muscle fibers. Histological sections stained with H&E revealed muscle infiltration of immune cells (red arrows) on day 2 (myolysis) and day 5 (early regeneration) following CTX injection. At d5, damaged myofibers were replaced by small newly formed myofibers with a centralized nucleus (white arrows). Twelve days upon CTX injury, myofibers with a centralized nucleus (white arrows) were enlarged and 'healthy' myofibers with a nucleus in the periphery appeared

(green arrows). PBS: healthy controls (PBS-injected), CTX-PL: CTX-injected and untreated, CTX-IBU: CTX-injected and ibuprofen-treated. Scale bar = 100μm.

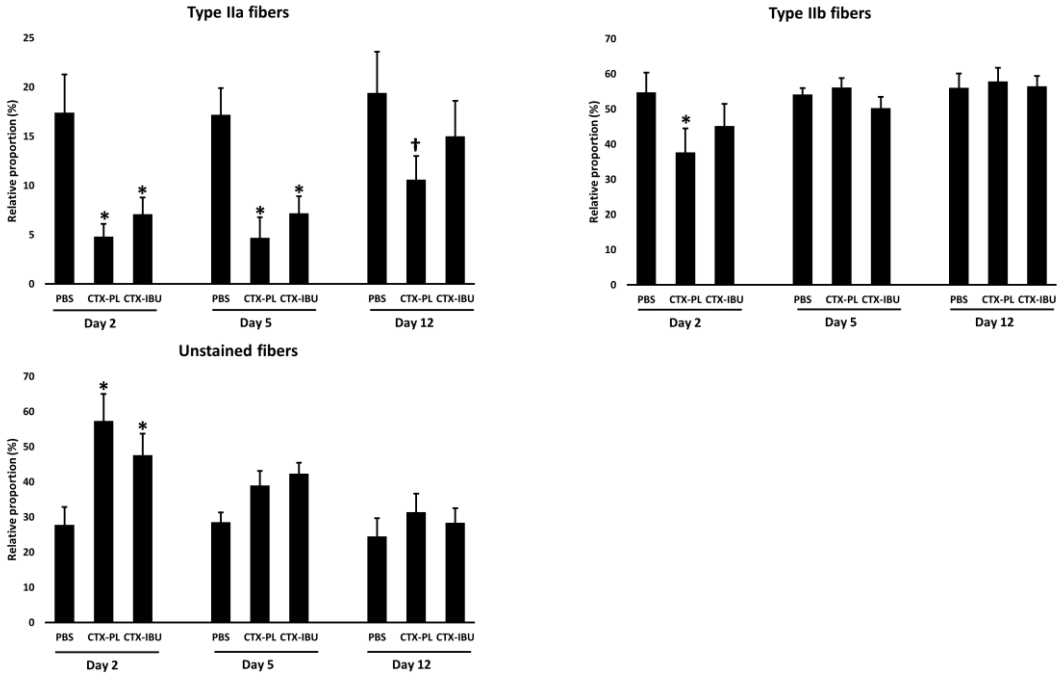

Fig. 3: Data are presented as average ± SEM for the relative contribution (as % of total fibers) of type IIa, type IIb and unstained (type IIx + immature) fibers of the muscle cross-section. PBS: healthy controls (PBS-injected), CTX-PL: CTX-injected and untreated, CTX-IBU: CTX-injected and ibuprofen-treated. *P < 0.05; † P = 0.05-0.1.

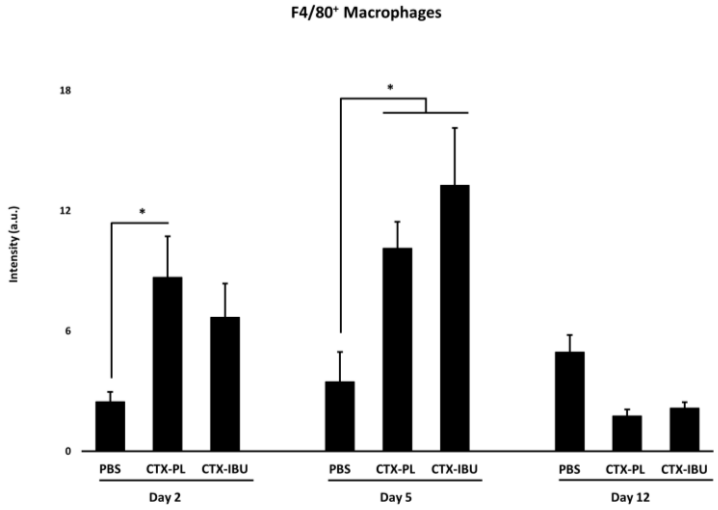

Fig. 4: Data are presented as average ± SEM for F4/80$^+$ macrophage infiltration. PBS: healthy controls (PBS-injected), CTX-PL: CTX-injected and untreated, CTX-IBU: CTX-injected and ibuprofen-treated. *p < 0.05.

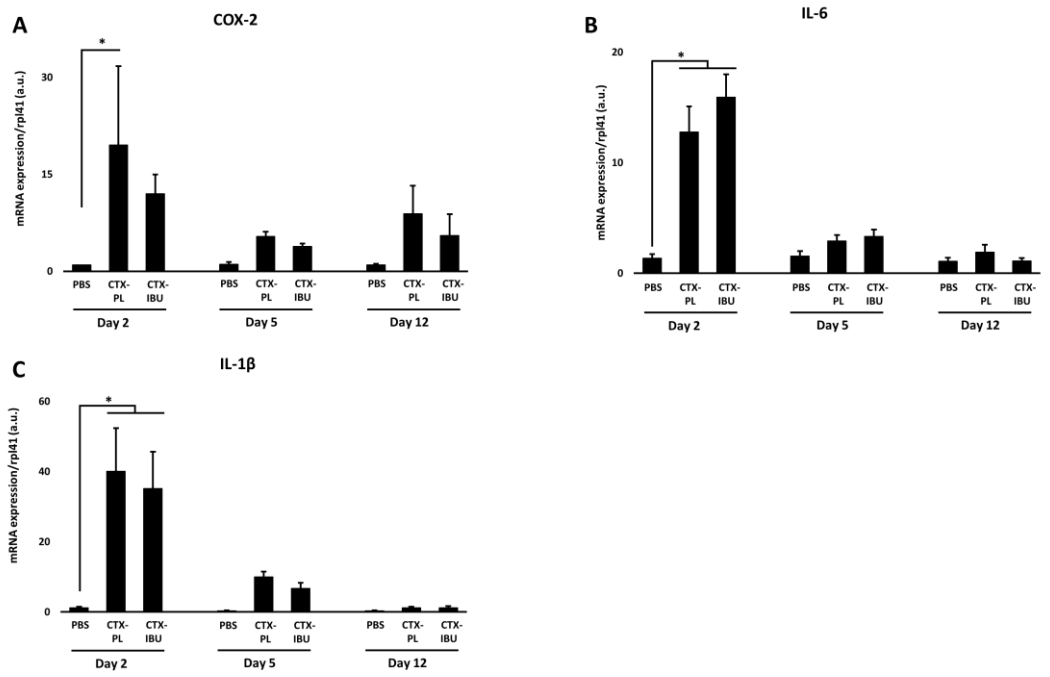

Fig. 5: Data are presented as average ± SEM for the mRNA expression of inflammatory markers, i.e. cyclooxygenase-2 (COX-2), interleukin-6 (IL-6) and interleukin-1β (IL-1β). PBS: healthy controls (PBS-injected), CTX-PL: CTX-injected and untreated, CTX-IBU: CTX-injected and ibuprofen-treated. *p < 0.05.

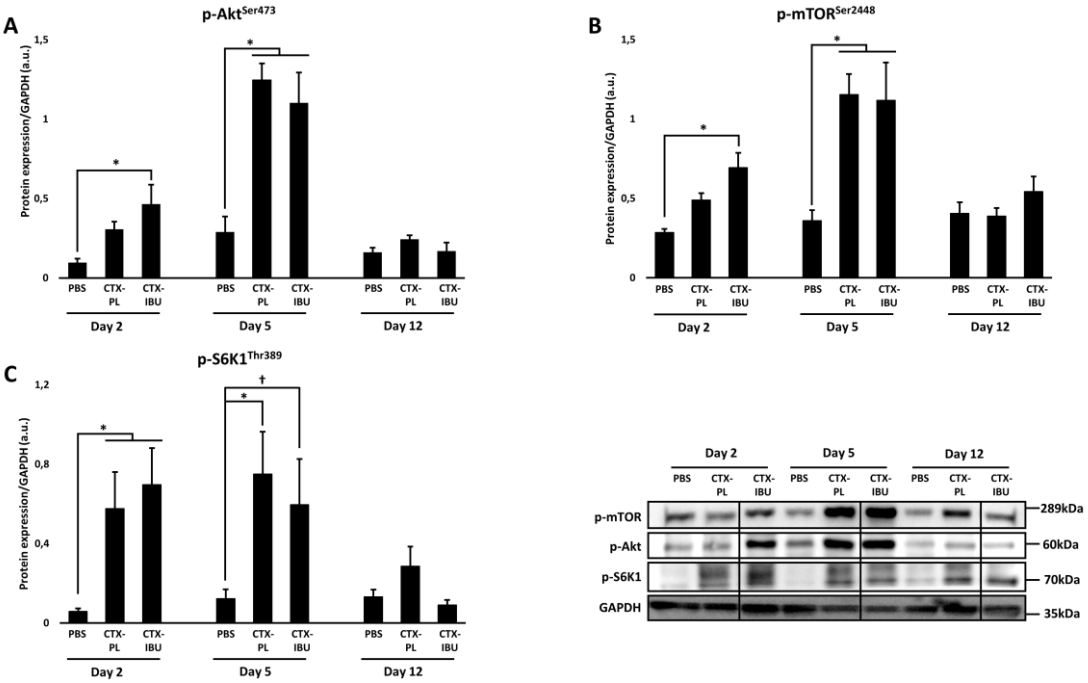

Fig. 6: Data are presented as average ± SEM for the protein expression of anabolic markers (mTORC1 pathway), i.e. phospho-Akt (p-Akt), p-mammalian target of rapamycin (p-mTOR) and p-ribosomal protein S6 kinase 1 (p-S6K1). PBS: healthy controls (PBS-injected), CTX-PL: CTX-injected and untreated, CTX-IBU: CTX-injected and ibuprofen-treated. *P < 0.05; † P = 0.05-0.1.

Primer sequences.

Gene	Forward	Reverse	Template	Product size
Cox-2	ATGGGTGTGAAGGGAAATAAGGA	CCTTGGGGGTCAGGGATGAA	NM_011198.4	92
Il-6	GACAAAGCCAGAGTCCTTCAGA	GTGACTCCAGCTTATCTCTTGGT	NM_001314054.1	75
*Il-1*β	ATGCCACCTTTTGACAGTGATG	GCAGCCCTTCATCTTTTGGG	NM_008361.4	72
Rpl41	ATGAGAGCGAAGTGGCGGAAG	CAGGGCAGAGGGACTGTTTTG	NM_018860.4	260

Table 1: COX-2: cyclooxygenase-2, IL: interleukin, Rpl41: ribosomal protein L41

Muscle fiber type composition.

Relative contribution (%)	Day 2			Day 5			Day 12		
	PBS	CTX-PL	CTX-IBU	PBS	CTX-PL	CTX-IBU	PBS	CTX-PL	CTX-IBU
Type I	0.04±0.03	0.04±0.03	0.04±0.04	0.02±0.02	0.10±0.08	0.16±0.11	0.06±0.05	0.06±0.04	0.05±0.04
Type IIa	17.4±3.9	4.8±1.3*****	7.1±1.7*****	17.2±2.7	4.7±2.1*****	7.2±1.7*****	19.4±4.2	10.6±2.4†	15.0±3.6
Type IIb	54.8±5.6	37.7±6.8*****	45.2±6.3	54.2±1.8	56.2±2.6	50.3±3.2	56.1±4.0	57.9±3.9	56.5±2.9
Unstained fibers	27.8±5.1	57.4±7.6*****	47.6±6.1*****	28.6±2.7	39.0±4.1	42.4±3.1	24.5±5.2	31.4±5.3	28.4±4.1

Table 2: Data are average ± SEM for relative fiber number (%) in the m. *Tibialis Anterior*. PBS: healthy controls (PBS-injected), CTX-PL: CTX-injected and untreated, CTX-IBU: CTX-injected and ibuprofen-treated. *P < 0.05; †P = 0.05-0.1 compared to PBS within same day following sacrifice.