The Journal of Physiology

NSAID ingestion augments training-induced muscle hypertrophy and differentially affects muscle mRNA expression, but not strength gains, in trained men

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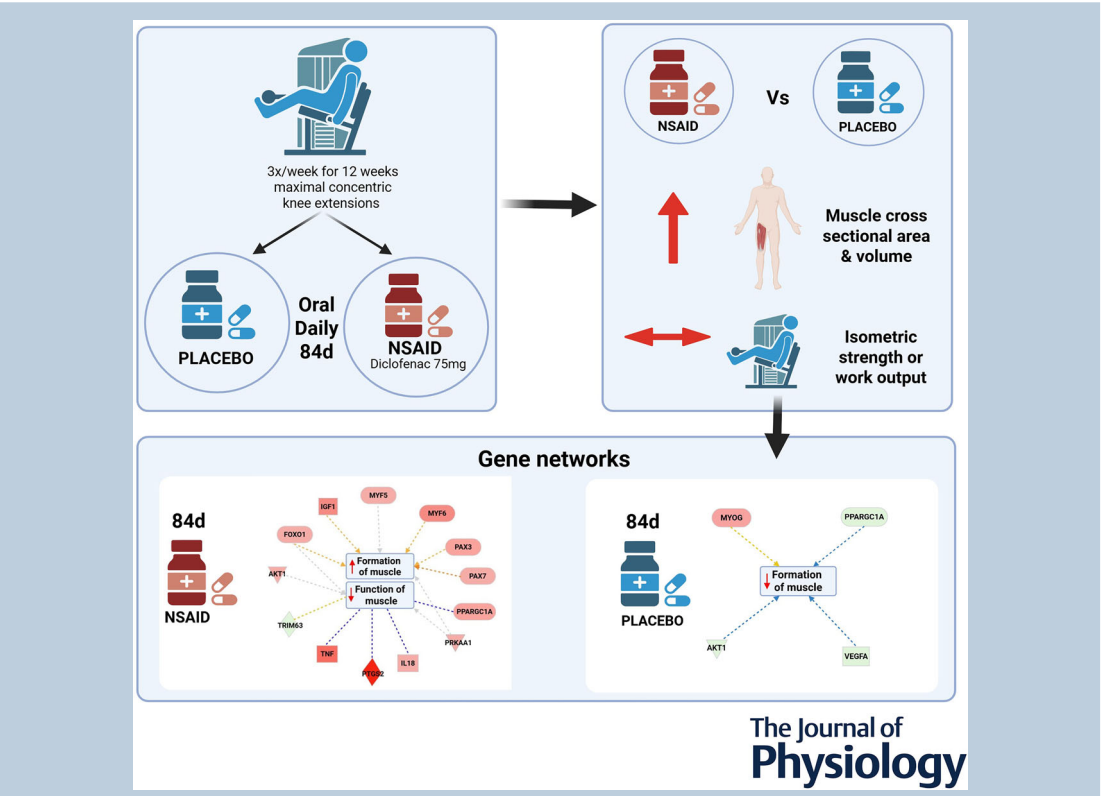
Handling Editors: Karyn Hamilton & Kevin Murach

The peer review history is available in the Supporting Information section of this article (https://doi.org/10.1113/JP289542#support-information-section).



Abstract figure legend Schematic outlining the impact of NSAID ingestion on resistance exercise training-induced changes in muscle morphology, function and gene networks relative to placebo ingestion in trained males.

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DOI: 10.1113/JP289542

Abstract Non-steroidal anti-inflammatory drugs (NSAIDs) are widely overused in sports. The temporal effects of combined NSAID consumption and resistance exercise training (RET) on muscle cross-sectional area (CSA), volume and targeted mRNA transcripts ($n = 93$) were quantified. Seventeen trained males (24.5 ± 1.1 years, body mass index (BMI) 24.2 ± 0.7 kg/m^2) consumed either placebo (PLA; $n = 8$) or diclofenac (75 mg, NSAID; $n = 9$) daily for 12 weeks and performed 3×30 maximal, isokinetic knee extensions in the non-dominant leg (90°/s) thrice each week. Quadriceps muscle CSA and volume were measured at baseline, 28 days and 84 days (3T MRI). Vastus lateralis biopsies were obtained at baseline, 24 h, 7 days, 28 days and 84 days for mRNA abundance measurements (RT-PCR microfluidic cards). Work output throughout RET was no different between groups. Muscle CSA was increased from baseline in both groups at 28 days (PLA 4.3 ± 2.5%, $P = 0.039$; NSAID 4.6 ± 3.7%, $P = 0.011$), but only in the NSAID group at 84 days (PLA 3.9 ± 0.8%, NSAID 8.6 ± 5.3%; $P < 0.001$; NSAID *vs*. PLA, $P = 0.038$), and was paralleled by muscle volume changes. RET increased isometric strength (∼40%–50%), but gains were no different between groups. Based on mRNA expression changes several cellular functions associated with muscle mass and metabolic regulation were altered in both groups throughout RET and were greater in NSAID at 28 and 84 days. NSAID intervention produced greater muscle hypertrophy than PLA, which occurred between 28 and 84 days of RET and was paralleled by more pronounced muscle mRNA changes. These collective events were not accompanied by greater strength gains, suggesting that using NSAIDs alongside RET may not be optimal for enhancing sports performance.

(Received 20 June 2025; accepted after revision 1 October 2025; first published online 24 October 2025)
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Key points

- Non-steroidal anti-inflammatory drug (NSAID) ingestion over 84 days of resistance exercise training increased muscle cross-sectional area and volume gains compared to placebo ingestion in young, trained male volunteers, and this occurred predominantly from day 28 to day 84 of training.
- In parallel with this, alterations in gene networks associated with a number of cellular functions linked to regulation of muscle mass and muscle metabolism were detected in the NSAID group relative to placebo.
- This greater resistance training-induced hypertrophy associated with NSAID ingestion was not accompanied by greater gains in isometric knee extensor strength or isokinetic work output during training compared to resistance training alone.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are available both by prescription and over the counter (OTC) and have been widely used for their anti-inflammatory and analgesic actions for many decades in multiple conditions (Baum et al., 1985; Connolly, 2003; Kean & Buchanan, 2005; Lipton et al., 1998). More than

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30 million people worldwide consumed prescription NSAIDs every day at the start of this century (Singh, 2000), and more than 15 million NSAID prescriptions were filled in 2014 in England alone (Davis & Robson, 2016), reflecting their widespread use. NSAIDs act by competitive inhibition of the cyclo-oxygenase (COX) enzyme, blocking the production of prostaglandins that contribute to inflammation and pain (Scholer et al., 1986).

NSAIDs have a history of use in sport at various levels. A study of Irish collegiate student-athletes reported 94% had used NSAIDs previously, and 64% used NSAIDs before or after practice and/or competition (O'Connor et al., 2019). From a sample of 129 athletes 68% reported using NSAIDs over the prior 12 months (Rudgard et al., 2019). Nearly a third of all Olympic athletes in the late 1990s to the 2000s reported NSAID use in the 3 days before data were collected from them, and in certain sports this was reported to be as high as 100% (Corrigan & Kazlauskas, 2003; Huang et al., 2006; Tsitsimpikou et al., 2009). Further it has been estimated that the magnitude of NSAIDs use in Olympic athletes is 6–10 times greater than in an age-matched population (Berglund, 2001). Indeed elite athletes are reported to be 3.6 times more likely to use NSAIDs than age-matched controls (Alaranta et al., 2006). NSAID use is also evident in the armed forces; in 2014, 82% of active-duty soldiers in the US army were prescribed NSAIDS (Walker et al., 2017), with the total number of NSAID users likely to be greater, as over-the-counter NSAIDs were not accounted for in the study.

NSAIDs are not included in the World Anti-Doping Agency list of performance-enhancing medications. Yet evidence suggests their misuse, despite well-documented cardiovascular, gastrointestinal and musculoskeletal adverse effects (Bhala et al., 2013; McGettigan & Henry, 2013; Warner et al., 1999). In the aforementioned study in collegiate athletes (O'Connor et al., 2019) 13.9% of respondents consumed NSAIDs over the recommended dosage, which in turn was 22.9% greater for OTC NSAIDs. Of those amateur athletes in the study by Rudgard et al. who took NSAIDs only a quarter were advised to do so by a pharmacist or doctor (Rudgard et al., 2019). Between 45% and 65% of participants in city marathons in Bonn and Berlin reported taking NSAIDs prior to racing (Kuster et al., 2013), even though only 11% had pain going into the race (Brune et al., 2009). Further despite a wide awareness campaign directed towards team physicians at the 2014 FIFA (Fédération Internationale de Football Association) World Cup the incidence of NSAID use did not reduce (Vaso et al., 2015).

Respondents in both amateur and elite sport reported taking these medications primarily to reduce pain and inflammation during training and competition and to hasten recovery from force loss and functional decline associated with delayed-onset muscle soreness (O'Connor et al., 2019; Rudgard et al., 2019; Tscholl et al., 2009). Acutely these purported benefits are assumed to improve performance during training and competition (Gorski et al., 2011; O'Connor et al., 2019), despite the fact that the evidence on the effectiveness of NSAIDs on both ameliorating perceived muscle soreness and aiding recovery from functional decline is equivocal (Connolly et al., 2003). Chronic NSAID use to relieve pain during training is perhaps undertaken with a view towards indirectly enhancing muscle strength and size adaptations by allowing for more work to be performed (Reynolds, 2017). Indeed over 50% of elite triathlete respondents in a study by Gorski et al. (2011) reported consuming NSAIDs in the 3 months prior to an event to alleviate soreness during training. At the amateur level NSAID use before practice to prevent pain was reported by 40% (O'Connor et al., 2019) to 50% (Rudgard et al., 2019) of student-athlete respondents. Some reviewers have even suggested that the reduction in perception of effort by relieving pain is a performance-enhancing effect of NSAIDs during both endurance and resistance exercise sessions (Holgado et al., 2018; Lundberg & Howatson, 2018). However no studies have examined whether work done during training is increased by chronic NSAID ingestion.

Studies have also investigated whether there is a direct physiological effect of NSAID administration on resistance exercise-induced muscular adaptations via mechanisms underlying hypertrophy and myogenesis. Trappe et al. reported postresistance exercise muscle protein synthesis (MPS) was suppressed by oral administration of 1200 mg ibuprofen over 24 h in young and healthy men compared to placebo ingestion (Trappe et al., 2002). Investigators have since demonstrated that NSAIDs inhibit satellite cell proliferation for up to 8 days after a bout of maximal eccentric exercise (Mackey et al., 2007; Mikkelsen et al., 2009), and blunt anabolic signalling in the hours following a bout of unaccustomed high-intensity resistance exercise (Markworth et al., 2014). More recently Lilja and colleagues reported that 1200 mg/day ibuprofen ingestion attenuated strength and hypertrophic responses to 8 weeks resistance training in young and healthy adults when compared to low-dose (75 mg/day) acetylsalicylic acid intervention (Lilja et al., 2018). However in contrast to these reports data from a more chronic intervention study in older participants (Trappe et al. 2011) demonstrated that NSAID administration enhanced training-induced muscle adaptations in a population whom age-related loss of muscle mass is a significant concern (Mitchell et al., 2012). Specifically Trappe et al. (2011), utilising a double-blind design, randomly assigned oral doses of either 4000 mg/day of paracetamol, 1200 mg/day of ibuprofen or placebo to healthy older volunteers (24 males, 12 females) who concurrently underwent

a 12 week progressive resistance training programme comprising three sessions a week. The investigators found that chronic NSAID ingestion alongside resistance training resulted in a ∼47% greater increase in quadriceps muscle volume compared to placebo, and a similar response was observed in the volunteers that ingested paracetamol.

In the context of this background the aim of the present study was to determine whether daily ingestion of the NSAID, diclofenac, would positively impact upon resistance exercise training-induced increases in muscle cross-sectional area (CSA), volume and strength over 12 weeks of intervention in young, trained men when compared to a blinded, placebo intervention. Furthermore we examined whether any outcomes observed were localised to a discernible time period of intervention and could be associated with NSAID-mediated changes in targeted muscle mRNA expression and/or training workload volume over time.

Methods

Participants

Seventeen healthy, non-smoking, non-vegetarian men (age: 24.5 ± 1.1 years; body mass index (BMI): 24.2 ± 0.7 kg/m^2) were recruited to this study. Participants were required to have participated in resistance exercise training or sports such as squash, football and basketball at least 2–3 days per week for a minimum of 2 years to minimise neural responses to resistance exercise (Moritani, 1993). The study was approved by the University of Nottingham Medical School Ethics Committee (ethics reference no: I 07 2011) in accordance with the Declaration of Helsinki, except from being registered as a clinical trial. Prior to taking part all participants provided informed, written consent, underwent a routine medical screening and completed a general health questionnaire. Participants were asked to refrain from taking potential anabolic supplements, that is protein and creatine, for the duration of the study but were allowed to continue with their regular training regimens apart from lower limb resistance training.

Exercise training protocol

A rehabilitation protocol consisting of a bout of 5 sets of 30 maximal isokinetic concentric knee extensions has been shown by our laboratory to be well tolerated and effective at increasing muscle mass and strength in previously untrained volunteers (Jones et al., 2004). In the present study participants trained thrice per week for 12 weeks and were not in an overnight fasted state for their training sessions. During each session they performed 5 bouts of 30 maximal isokinetic concentric contractions of the knee extensors in the non-dominant leg on an isokinetic dynamometer (HUMAC Norm, CSMi Solutions, MA, US) at an angular velocity of 90°/s with 3 min rest between bouts, which ensures that all muscle fibres of the quadriceps muscle group are recruited (Tesch et al., 1990). Volunteers received real-time visual feedback on their performance and verbal encouragement throughout each training session to expedite maximum effort being achieved. All volunteers completed 150 contractions per session and 5400 contractions over the 12 week training period.

NSAID intervention

This study was a randomised, placebo-controlled, double-blind 12-week experiment (Fig. 1). Participants were assigned to either a control intervention group who were given placebo in the form of lactose capsules (PLA; n = 8, 24.3 ± 1.7 years, BMI 23.5 ± 0.9kg.m^{-2}) or an intervention group who were administered diclofenac capsules (NSAID; 75 mg diclofenac + 15 mg lansoprazole; n = 9, 24.8 ± 1.4 years, BMI 24.8 ± 0.9kg.m^{-2}). Both treatments were ingested daily for 12 weeks alongside the training protocol. Both diclofenac and placebo capsules were obtained from the Nottingham University Hospitals (NUH) Pharmacy Department – Clinical Trials Services. Volunteers were randomised into two groups using an online randomisation tool (http://www.randomization.com/). NUH Pharmacy Department determined which of the two groups was PLA or NSAID, and blinded the investigators to the treatment, which was achieved by providing both diclofenac and placebo capsules in the same brown bottles, rendering them indistinguishable. Table 1 shows that both study groups were well matched with respect to age, height, weight, muscle CSA/volume and strength at baseline.

Diclofenac, chemically known as 2-(2,6-dichloranilino) phenylacetic acid, has a strong preference for inhibition of COX-2 isoenzymes as opposed to COX-1, demonstrating a COX-1/COX-2 selectivity ratio of 29 *ex vivo* in whole blood (Hunter et al. 2015; Warner et al. 1999). It was selected for use in this study because of its greater potency than comparable NSAIDs, allowing lower doses to be consumed and thereby minimising risk of negative gastrointestinal events (Henry et al. 1996; van Walsem et al. 2015). Participants were advised to take the tablets with breakfast, and always before training, in combination with a proton pump inhibitor, lansoprazole, to prevent chronic stomach irritation from the NSAID. Tablets were given to the subjects every 4 weeks. They were asked to return the bottles, so compliance could be determined by the number of tablets remaining after the 4 week period. Potential side effects of the diclofenac consumption

Table 1. Participant characteristics at baseline

Variables	PLA	NSAID
Age (years)	25.9 ± 4.61	26.4 ± 4.03
Height (m)	1.83 ± 0.04	1.80 ± 0.03
Body mass (kg)	78.7 ± 8.40	80.6 ± 9.69
BMI (kg.m^{-2})	23.6 ± 2.49	24.8 ± 2.84
Isometric strength (kg)	51.6 ± 18.57	50.2 ± 19.57
Quadriceps muscle CSA (cm^2)	88.9 ± 12.3	85.5 ± 8.3
Quadriceps muscle volume (cm^3)	2520.8 ± 388.5	2397.6 ± 298.6

Note: All values expressed as mean ± SD. Age, height, body mass, body mass index (BMI), isometric strength, quadriceps muscle cross-sectional area (CSA) and quadriceps muscle volume are shown for the group consuming placebo (PLA; n = 8) and the group consuming NSAIDs (NSAID; n = 9).

were monitored by monthly blood sampling for hepatic (alanine aminotransferase, ALT) measures.

Total work output during isokinetic knee extensor exercise training

Total isokinetic work output per week of training was calculated as the work done during all 450 maximal contractions performed each week. Total output over 12 weeks was calculated as sum of the work done from all exercise sessions during the protocol.

Knee extensor isometric strength

Isometric strength (best of 3 attempts) of the knee extensor muscles was measured with the knee flexed at 90° during a static maximal voluntary contraction using an isometric knee extension unit (Medical Physics, University of Nottingham, UK) at baseline (BL), 28 days and 84 days (Fig. 1). All measurements were made at the same time of day.

MRI mid-thigh muscle CSA and thigh muscle volume measurements

Quadriceps muscle CSA and volume measurements were performed in the rested state at BL, 28 days and 84 days (≥72 h from the previous training session). We hoped that reporting both end-point measures would allow some insight into the assessment of hypertrophy (increase in whole muscle size) and potential changes in muscle force-generating capacity. For this axial plane scans of the thigh were taken using a 3 Tesla MRI scanner (GE 3T 750 Discovery). A T1 Fast Spin Echo protocol was used (repetition time 600 ms, echo time 15 ms, number of excitations 2, field of view 22 mm, slice thickness 10 mm, no gap between slices). Participants were asked to lie supine on the magnetic resonance imaging (MRI) bed.

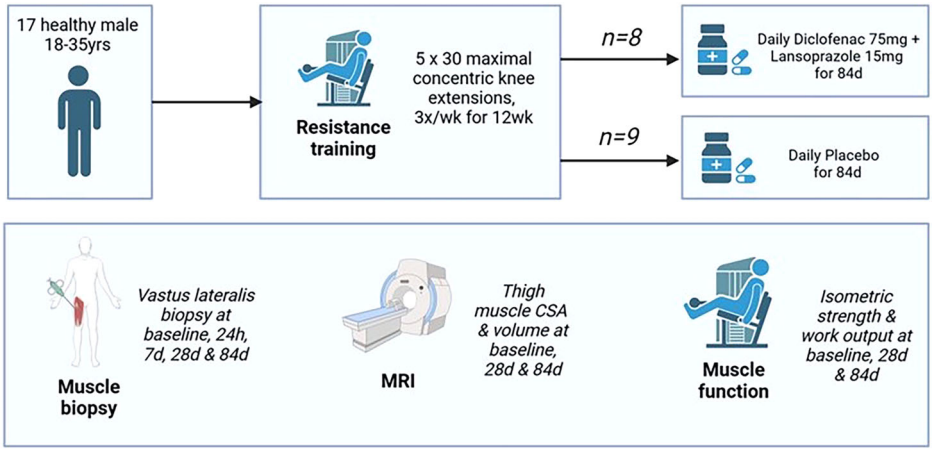

Figure 1. Study protocol
Subjects performed 5×30 maximal isokinetic concentric knee extensions 3 times per week for 12 weeks in the non-dominant limb with either daily ingestion of placebo or the NSAID diclofenac. Muscle biopsies were taken at baseline (BL), 24 h, 7 days, 28 days and 84 days. Isometric strength was measured at BL, 28 days and 84 days. Magnetic resonance imaging (MRI) of the thigh was performed at BL, 28 days and 84 days.

Axial plane scans ($n = 48$) along the entire length of the quadriceps muscle were obtained to determine anatomical CSA (ACSA). The ACSA represents the changes in the peak value ($ACSA_{peak}$) at the middle of the femur length. Using the axial scans the contours of the whole quadriceps muscle group of each MRI scan were digitised using Osirix image analysis software (Pixmeo, Geneva, Switzerland). Subsequently quadriceps muscle volume was calculated as follows:

$$\text{Volume quads}\left(\text{cm}^3\right) = \sum \text{ACSA}_{\text{peak}} \times \text{slice thickness}$$

(Franchi et al., 2014)

Muscle sample collection

Vastus lateralis muscle biopsies were obtained in the resting state at BL, 24 h and a minimum of 72 h from the previous exercise session at 7, 28 and 84 days (Fig. 1). Volunteers attended the laboratory in a fasted state, having abstained from alcohol and strenuous exercise for 48 h prior. Muscle samples were obtained from both legs using the Bergstrom percutaneous needle biopsy technique (Bergstrom, 1975), and samples were immediately snap frozen and stored in liquid nitrogen for further analysis.

Muscle mRNA expression

RNA was extracted from ∼30 mg snap-frozen muscle from all but 4 biopsy samples (i.e. 78 biopsies in total due to tissue availability), as previously described (Constantin et al., 2007). First-strand cDNA was synthesised from 1 μg of total RNA, using Superscript III reverse transcriptase (Invitrogen Ltd, Paisley, UK) and random primers (Promega, Southampton, UK) and stored at −80°C until analysis. TaqMan low-density arrays were performed using an ABI PRISM 7900HT sequence detection system, and data were analysed using SDS 2.1 software (Applied Biosystems, Waltham, Massachusetts, USA). Data were further analysed using RQ Manager software (Applied Biosystems), where the threshold level was normalised across all plates before Ct values were calculated for each gene target and sample. Relative quantification of mRNAs of interest was measured using the $2^{\Delta\Delta Ct}$ method with hydroxymethylbilane synthase (HMBS) as the endogenous control, with the mean of the baseline sample used as the calibrator. Mean Ct values for HMBS were no different between PLA and NSAID (data not shown). A total of 93 transcripts were targeted for analysis in the present study (Table 2 for list of genes). Transcripts identified as being altered following acute exercise in array studies involving healthy male volunteers were selected for inclusion in the present study (Chen et al., 2003; Higbie et al., 1996) along with muscle transcripts seen to be differentially expressed in healthy male volunteers over the course of 10 weeks of concentric resistance training (Murton et al., 2014). Based on these studies approximately 50 genes were selected that were linked to cell growth and regulation, DNA damage, stress responses, energy metabolism, inflammation, extracellular matrix and muscle differentiation and signalling. Additionally mRNA expression of myogenic transcription/regulatory factors, such as myoblast determination protein 1 (MyoD), myogenin (MYOG), myostatin (MSTN), myogenic factor 5 (MYF5), was selected for inclusion. Further genes of interest, known to be differentially regulated in muscle inflammatory states and linked to muscle mass regulation, were also included (Crossland et al., 2008; Greenhaff et al., 2008; Mallinson et al., 2009), namely AKT serine/threonine kinase 1 (AKT1), forkhead box protein O 1 (FOXO1), protein tyrosine kinase 2 (PTK2), pyruvate dehydrogenase kinase 4 (PDK4), DNA (cytosine-5)-methyltransferase (Met1A), cathepsin-L, muscle-specific ligases atrophy F-box protein (MAFbx), muscle RING-finger protein-1 (MuRF1) and proteasomal alpha subunit 1 (PSMA1).

Ingenuity pathway analysis

To associate a biological function to the identified probe sets Ct values were uploaded using Ingenuity Pathway Analysis (IPA) software (Redwood City, CA, USA) for pathway analysis of gene expression data. IPA is a commercial web-based software application that enables analysis, integration and understanding of data from gene expression, miRNA and single nucleotide polymorphism (SNP) microarrays, as well as metabolomics, proteomics and RNAseq experiments. The outcome of IPA analysis performed in this study could, on the one hand, appear biased because the input was the result of an array analysis of 93 preselected genes, as opposed to an unbiased analysis of gene expression on a global level. However the aim of the study was to quantify the impact of NSAID ingestion on these defined target genes in response to resistance exercise training and was therefore not intended to be hypothesis-generating in nature (typical of untargeted analysis). Furthermore the overall outcome of each IPA analysis (e.g. upstream regular analysis, cellular function, activation status) was predicted by calculating a regulation Z-score and an overlap *P*-value, which were based on the number of regulated target genes' function, size change and direction of expression, and their agreement with the IPA database, constructed on manual curated literature searches.

Muscle protein expression measurements

Western blotting was used to quantify target protein expression of inflammatory proteins interleukin 6 (IL-6),

Table 2. List of genes used on low-density RT-PCR array microfluidic cards (Applied Biosystems Inc., Foster City, CA, USA)

Symbol	Gene name
ABLIM2	Actin-binding LIM protein family member 2
ACTC1	Actin, alpha, cardiac muscle 1
ACTN3	Alpha-actinin-3
ACVRC1	Activin A receptor 1
ADORA1	Adenosine A1 receptor
AKT1	Serine-threonine protein kinase
ANKRD1	Ankyrin repeat domain 1
ANKRD2	Ankyrin repeat domain 2
ASB5	Ankyrin repeat and SOCS box containing 5
ATF3	Activating transcription factor 3
CALCR	Calcitonin receptor
CD34	CD34
CDH15	Cadherin 15
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
CLEC3B	C-type lectin domain family 3, member B
CTSL1	Cathepsin L
CYR61	Cysteine-rich angiogenic inducer 61
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1
EIF2B	Eukaryotic translation initiation factor 2B, subunit 2 beta
FABP3	Fatty acid binding protein 3, muscle and heart
FBXO32	F-box protein 32
FIGF	Cellular oncogene c-fos
FOXO1	Forkhead transcription factor 1A
FOXO3	Forkhead transcription factor 3
GADD45a	Growth arrest and DNA damage-inducible protein alpha
GADD45b	Growth arrest and DNA damage-inducible protein
GEM	GTP-binding protein overexpressed in muscle
GSK3b	Glycogen synthase kinase 3 beta
HDAC9	Histone deacetylase
HEMk1	HemK methyltransferase family member 1
HGF	Hepatocyte growth factor
HIF1a	Hypoxia inducible factor 1, alpha subunit
HSPA1a	Heat shock protein (70 kDa) 1A
HSPA1b	Heat shock protein (70 kDa) 1B
IFRD1	Interferon-related developmental regulator 1
IGF1	Insulin-like growth factor 1
IGJ	JCHAIN
IL-18	Interleukin 18
IL-6	Interleukin 6
ITGA10	integrin, alpha 10
JKAMP	Mitogen-activated protein kinase 8
Jun	c-jun
MAFF	MAF bZIP transcription factor F
MAP3K12	Mitogen-activated protein kinase kinase kinase 12
MDF1	MAD (yeast mitosis arrest deficient) related
MET	MET proto-oncogene
MSTN	Myostatin
MYC	MYC proto-oncogene
MYF5	Myogenic factor 5
MYF6	Myogenic factor 6
MYH1	Myosin heavy chain 1
MYH2	Myosin heavy chain 2
MYH3	Myosin heavy chain 3

(*Continued*)

Table 2. (Continued)

Symbol	Gene name
MYH4	Myosin heavy chain 4
MYH7	Myosin heavy chain 7
MYOG	Myogenin
NEDD4	Neural precursor cell expressed, developmentally down-regulated 4-like,
NFKb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
PAX3	Paired box 3
PAX7	Paired box 7
PDK2	Pyruvate dehydrogenase kinase, isoenzyme 2
PDK4	Pyruvate dehydrogenase kinase, isoenzyme 4
PDP1	Pyruvate dehydrogenase phosphatase catalytic subunit 1
PFK1	Phosphofructokinase 1
PIK3R1	Phosphatidylinositol 3-kinase, regulatory 1 (p85 alpha)
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PRKAA1	Protein kinase AMP-activated catalytic subunit α 1
PSMA1	Proteasome subunit alpha 1
PTGS1	Cyclo-oxygenase 1
PTGS2	Cyclo-oxygenase 2
PTK2	Focal adhesion kinase
Rabb15	Member RAS oncogene family
RPS6	Ribosomal protein S6
RPS6KA	Ribosomal protein S6 kinase
RRPTOR	Regulatory associated protein of MTOR, complex 1
RRAD	Ras-related glycolysis inhibitor and calcium channel regulator
SCD	Stearoyl-coenzyme A desaturase 1
SIRT1	Sirtuin 1
SREBF2	Sterol regulatory element binding protein
SRF	Serum response factor
TGF1b	Transforming growth factor, beta 1
TNC	Tenascin C
TNF-α	Tumour necrosis factor alpha
TRIM63	Tripartite motif containing 63
TSC2	Tuberous sclerosis 2
Tubb2A	Tubulin beta 2A
Tubb	Tubulin beta
TXNIP	Thioredoxin-interacting protein
VEGFA	Vascular endothelial growth factor alpha
YWZHA	Tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase
ZFP36	Zinc finger protein 36, C3H type

tumour necrosis factor α (TNF-α) precursor and mature TNF-α from muscle samples obtained at BL, 24 h, 7, 28 and 84 days. Protein was extracted from approximately 30 mg snap-frozen tissue using a method modified from Blough et al. (1999). Homogenisation of samples was carried out in 50 mM Tris buffer pH 7.5, and protease and phosphatase inhibitors (Sigma-Aldrich, UK) were added. Samples were then centrifuged, and the supernatant with cytosolic protein fraction collected. The Bradford assay was used to measure protein content. Protein samples were run on a 4%–12% Bis-tris acrylamide gel (Invitrogen, Paisley, UK) at 200 V for 1 h. They were then transferred to a polyvinylidene difluoride membrane for 1 h 45 min. This transfer was checked with Ponceau S staining and then blocked in 5% BSA-TBS-Tween. Probing of membranes was carried out with TNF-α (1:1000, Cell Signalling, USA) and IL-6 (1:1000, Cell Signalling) primary antibodies overnight at 4°C. TBS-Tween was used to wash the membrane, which was then incubated with the secondary antibody anti-rabbit IgG (H+L) (DyLight 680 Conjugate, Cell Signalling). Blots were scanned, and bands were identified using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA). Density volume was adjusted by the subtraction of the local background and then normalised using actin (1:1000; Sigma-Aldrich).

Statistical analysis

All values are presented as mean ± SD (and individual values in Figs 2 and 3), unless otherwise stated, with significance accepted at $P < 0.05$ level. Exact P-values are presented except when $P < 0.001$. Normality was tested using the Shapiro-Wilks test. Where data were not normally distributed, values were log transformed, and parametric statistical tests used. Comparison of mean values between PLA and NSAID groups was performed using a repeated-measures two-way ANOVA (time and treatment as independent factors) with Tukey's or Sidak's *post hoc* test analysis where appropriate (GraphPad Prism 10). To identify the statistical difference in total work output between PLA and NSAID groups over the entire exercise protocol a paired sample, two-tailed t test was used (GraphPad Prism 10).

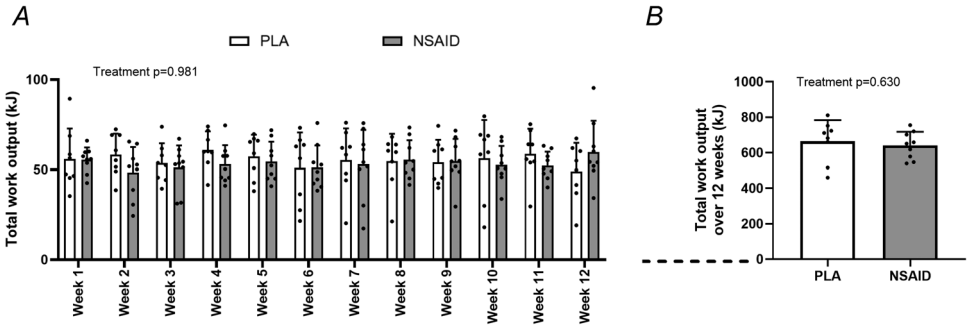

Figure 2. Muscle function
A, total work output (kJ) per week; (*B*) total work output (kJ) over 12 weeks in the groups consuming placebo (PLA, $n = 8$) and diclofenac (NSAID; $n = 9$). Values are expressed as mean ± SD and individual values.

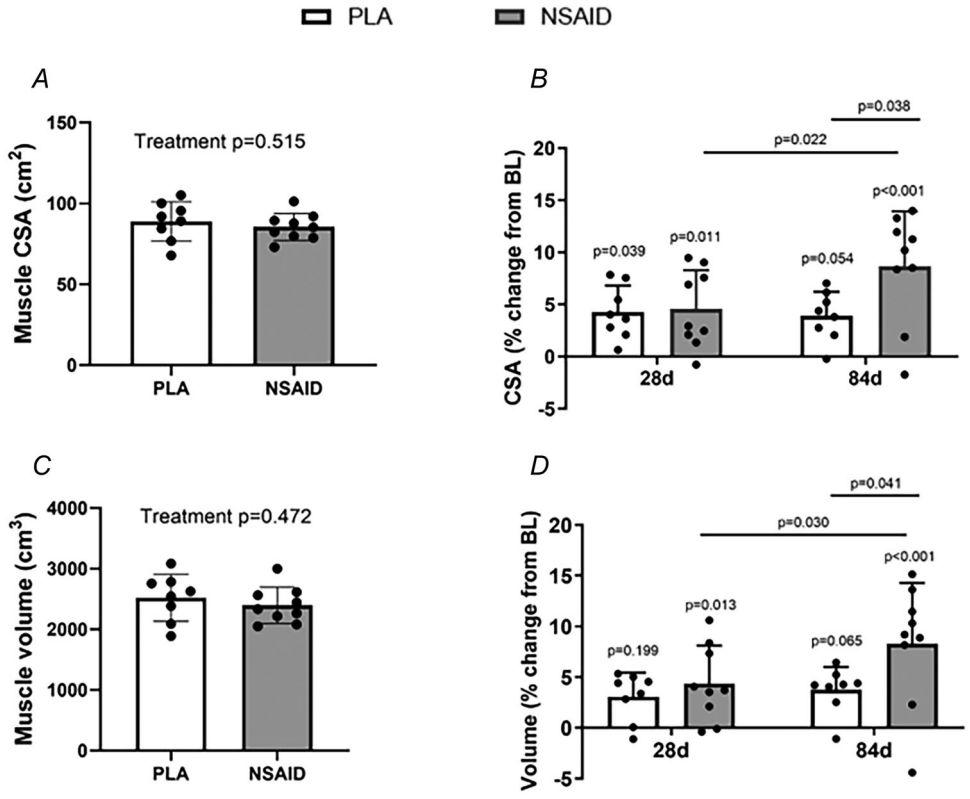

Figure 3. Muscle morphology
A, absolute mid-thigh quadriceps muscle cross-sectional area (cm^2) at baseline. *B*, percentage change from baseline in mid-thigh quadriceps muscle cross-sectional area (%) at 28 days and 84 days of training. *C*, absolute quadriceps muscle volume (cm^3) at baseline and (*D*) percentage change from baseline in quadriceps muscle volume (%) at 28 days and 84 days of training in the groups consuming placebo (PLA; $n = 8$) and diclofenac (NSAID; $n = 9$). Baseline is set at 0. Values are expressed as mean ± SD and individual values.

Gene transcript analysis and interpretation using IPA utilised the comprehensive, manually curated content of the Ingenuity Knowledge Base. Statistical significance of mRNA expression was determined from the fold-change data for each timepoint using a paired sample *t* test, and the $\log^2$ fold difference for each gene and its associated *P*-value were uploaded to IPA software (Redwood City). Powerful algorithms identified regulators, relationships, mechanisms, functions and pathways relevant to changes observed in the dataset uploaded to IPA. To control for an enrichment of false-positive results (type II errors) when undertaking multiple comparisons we used Bonferroni's corrected P-value < 0.05.

Results

None of the participants taking the NSAID presented with any side effects associated with this medication (i.e. stomach irritation/pain or abnormal ALT levels) during the course of the study.

Muscle function during training

Total work output per week during training was no different between groups ($P = 0.630$, Fig. 2*A*), and neither was the total amount of work done over the 12 weeks of training ($P = 0.981$, Fig. 2*B*).

Isometric strength of the knee extensors increased over time ($P = 0.010$). By the end of training isometric strength had increased from baseline in both groups by ∼40%–50% on average, but the increase was significant in only the NSAID group (PLA 44.7 ± 40.0%, $P = 0.132$, NSAID 50.8 ± 52.2%, $P = 0.049$). Furthermore, there was no difference in isometric strength gains when comparing PLA and NSAID ($P = 0.998$).

Muscle CSA and volume

Quadriceps muscle CSA was no different between groups at baseline (Fig. 3*A*). Quadriceps muscle CSA increased above baseline at 28 days in both the PLA (4.3 ± 2.5%, $P = 0.039$) and the NSAID groups (4.6 ± 3.7%, $P = 0.011$, Fig. 3*B*). There was a trend for muscle CSA to remain increased above baseline in the PLA group at 84 days (3.9 ± 2.3%, $P = 0.054$), but this was no different to the increase recorded at 28 days ($P = 0.999$). However muscle CSA continued to increase after 28 days in the NSAID group, such that the magnitude of increase from baseline at 84 days was 8.6 ± 5.3% ($P < 0.001$). This increase was greater than the increase recorded in the PLA group at 84 days ($P = 0.038$).

Quadriceps muscle volume was no different between groups at baseline (Fig. 3*C*). Quadriceps muscle volume increased above baseline at 28 days in the NSAID group (4.4 ± 3.8%, $P = 0.013$) but not in the PLA group (3.1 ± 2.4%, $P = 0.199$; Fig. 3*D*). Muscle volume on average increased above baseline in the PLA group at 84d, but this did not reach statistical significance (3.8 ± 0.8%, $P = 0.065$). Similar to muscle CSA muscle volume continued to increase after 28 days in the NSAID group such that the increase in volume from baseline at 84 days was 8.3 ± 6.0% ($P < 0.001$). This increase was greater than the PLA group at 84 days ($P = 0.041$).

Muscle mRNA expression

Ingenuity pathway analysis. Table 3 shows the differential expression of targeted gene transcripts in the groups consuming placebo and diclofenac from baseline at 24 h, 7 days, 28 days and 84 days. Figure 4 depicts those cellular functions most altered from baseline in the PLA (Fig. 4*A*) and NSAID (Fig. 4*B*) groups at 24 h, 7 days, 28 days and 84 days. The *x*-axis displays cellular functions most affected, whereas the *y*-axis displays the log of the *P*-values. The *P*-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The -log of *P*-value was calculated using Fisher's exact test (right-tailed).

Overall, in the PLA group (Fig. 4*A*), IPA detected that the most altered cellular functions [–log (*P* value) > 4.0], in descending order of magnitude, were those associated with skeletal and muscular development and function, organismal injuries and abnormalities, lipid metabolism, carbohydrate metabolism, skeletal and muscular disorders and tissue development. Figure 4 also illustrates that in the main, in the PLA group, the largest magnitude of response to exercise training occurred at 24 h and 7 days. However this early response to training had waned by 28 and 84 days for all cellular functions.

In the NSAID group (Fig. 4*B*) IPA detected that the most-altered cellular functions [–log (*P* value) > 7.5] were the same as those in the PLA group, albeit in a different descending order of magnitude. Unlike the PLA group, however, the large response to training at 24 h and 7 days was, in the main, maintained in magnitude at 28 days and 84 days in the NSAID group for all cellular functions.

Figure 5*A*–*C* shows muscle mRNA networks for the cellular function 'skeletal and muscular system development and function' in Fig. 4, as an example to illustrate how any cellular function identified as being altered in Fig. 4 was influenced by resistance training in PLA and NSAID interventions. Each figure depicts the most differentially regulated mRNAs from baseline (outer ring), and the cellular events predicted to result from these collective mRNA changes in NSAID and PLA groups at a specific time point in the intervention. Figure 5*A* relates to 'skeletal and muscular development and function' in the

Table 3. Differential expression of targeted gene transcripts in the groups consuming placebo (PLA; *n* = 8) and diclofenac (NSAID; *n* = 9) from baseline at 24 h, 7 days (7 d), 28 days and 84 days of training

Gene name	PLA								NSAID							
	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d
	Fold change from mean baseline				*P*-value				Fold change from mean baseline				*P*-value			
ABLIM2	1.93	1.47	1.08	0.78	0.40	0.50	0.27	0.53	5.27	2.21	1.62	1.64	0.11	0.33	0.60	0.00
ACTC1	34.85	4.46	5.63	9.10	0.02	0.03	0.96	0.28	39.85	32.06	15.87	8.23	0.12	0.03	0.03	0.84
ACTN3	2.08	0.93	1.63	1.25	0.28	0.25	0.41	0.63	7.28	4.55	1.72	5.28	0.90	0.83	0.49	0.81
ACVRC1	3.24	3.72	1.91	1.41	0.08	0.71	0.40	0.36	9.01	5.67	5.86	5.68	0.03	0.05	0.16	0.18
ADORA1	2.45	3.20	2.12	2.26	0.07	0.02	0.40	0.44	5.38	6.64	3.81	2.95	0.14	0.03	0.19	0.36
AKT1	1.89	1.55	1.95	0.88	0.27	0.41	0.25	0.63	4.41	2.40	3.02	2.45	0.07	0.78	0.01	0.70
ANKRD1	11.28	12.30	4.75	1.40	0.18	0.50	0.55	0.13	11.86	17.29	8.48	10.89	0.05	0.40	0.13	0.36
ANKRD2	6.31	1.80	1.28	1.41	0.13	0.46	0.59	0.48	4.24	2.22	1.77	1.42	0.51	0.56	0.74	0.04
ASB5	6.66	2.46	1.42	1.71	0.20	0.09	0.76	0.43	5.10	1.59	1.66	0.78	0.63	0.66	0.98	0.02
ATF3	2.86	2.13	1.51	2.04	0.28	0.25	0.73	0.14	2.09	2.09	9.31	1.69	0.31	0.67	0.35	0.22
CALCR	1.22	1.71	1.79	1.56	0.99	0.19	0.38	0.11	0.98	2.16	1.77	1.93	0.03	0.94	0.62	0.30
CD34	1.38	2.01	1.81	1.72	0.53	0.10	0.30	0.06	0.86	1.55	1.90	1.42	0.02	0.60	0.68	0.12
CDH15	1.54	2.53	1.34	1.70	0.97	0.17	0.30	0.09	2.32	2.65	1.15	1.95	0.28	0.88	0.20	0.05
CEBPB	2.10	1.68	1.42	1.75	0.40	0.29	0.95	0.56	1.37	1.21	1.10	1.60	0.12	0.37	0.52	0.16
CLEC3B	1.07	3.58	3.87	1.63	0.96	0.01	0.27	0.15	0.73	2.26	5.96	1.86	0.01	0.98	0.20	0.16
CTSL1	6.02	4.06	1.57	2.04	0.06	0.01	0.94	0.29	1.87	1.60	1.36	0.89	0.20	0.71	0.86	0.04
CYR61	5.56	5.99	3.02	6.17	0.03	0.00	0.06	0.00	2.69	2.85	3.31	4.49	0.55	1.00	0.39	0.64
DNAJB4	2.25	2.85	2.04	1.25	0.44	0.14	0.80	0.62	4.34	3.70	2.71	2.91	0.29	0.36	0.02	0.58
EFEMP1	3.40	8.88	1.46	2.02	0.03	0.00	0.63	0.21	2.26	5.92	11.59	2.66	0.43	0.28	0.07	0.52
EIF2B	1.28	1.31	1.17	1.12	0.51	0.93	0.60	0.41	2.48	2.08	1.58	2.30	0.88	0.21	0.58	0.58
FABP3	1.93	1.23	1.68	1.13	0.91	0.51	0.66	0.80	3.03	2.11	1.72	2.22	0.67	0.18	0.90	0.39
FBXO32	2.07	1.95	1.79	2.70	0.26	0.13	0.68	0.26	1.27	1.13	1.52	0.77	0.10	0.38	0.51	0.04
FIGF	1.07	2.39	1.00	1.43	0.37	0.38	0.48	0.26	1.49	3.35	1.42	2.59	0.05	0.57	0.77	0.70
FOXO1	1.57	1.46	1.18	1.60	0.32	0.16	0.40	0.04	1.39	2.14	1.43	1.68	0.01	0.93	0.94	0.05
FOXO3	1.53	1.46	1.33	1.39	0.93	0.87	0.60	0.20	2.42	2.28	1.58	2.36	0.45	0.28	0.37	0.58
GADD45a	2.52	4.59	1.26	1.53	0.13	0.04	0.73	0.24	25.93	11.73	2.93	12.35	0.68	0.58	0.42	0.95
GADD45b	4.36	3.12	2.21	1.62	0.02	0.03	0.36	0.06	2.81	1.81	2.11	1.51	0.96	0.84	0.63	0.23
GEM	1.86	5.68	2.14	5.26	0.26	0.65	0.43	0.07	3.25	3.17	4.30	4.28	0.79	0.62	0.10	0.23
GSK3b	1.25	1.07	1.15	1.44	0.72	0.75	0.96	0.19	1.04	1.17	0.94	1.04	0.07	0.27	0.44	0.03
HDAC9	1.96	1.59	1.58	1.46	0.47	0.14	0.78	0.28	1.82	1.46	1.33	1.10	0.19	0.44	0.86	0.03
HEMk1	1.44	1.72	1.44	1.23	0.34	0.08	0.99	0.45	1.26	2.08	1.61	1.95	0.06	0.96	0.78	0.37
HGF	2.53	19.38	2.92	2.75	0.04	0.03	0.32	0.04	4.58	5.17	7.26	3.31	0.38	0.02	0.01	0.74
HIF1a	1.76	5.68	2.08	1.61	0.05	0.16	0.50	0.06	3.72	4.08	3.94	2.91	0.57	0.05	0.00	0.55
HSPA1a	3.12	2.09	1.76	2.23	0.18	0.10	0.76	0.36	1.56	1.57	1.44	0.93	0.17	0.69	0.94	0.04
HSPA1b	2.16	1.94	1.69	1.33	0.50	0.78	0.80	0.71	3.51	2.61	1.93	2.53	0.48	0.66	0.71	0.83
IFRD1	1.31	1.62	1.12	1.03	0.65	0.19	0.74	0.27	1.56	1.29	1.89	1.24	0.16	0.11	0.92	0.04

(*Continued*)

Table 3. (Continued)

Gene name	PLA								NSAID							
	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d
	Fold change from mean baseline				*P*-value				Fold change from mean baseline				*P*-value			
IGF1	5.78	2.47	2.18	2.35	0.46	0.49	0.47	0.02	2.51	3.14	4.41	3.54	0.31	0.37	0.00	0.30
IGJ	3.22	16.18	2.24	2.17	0.11	0.03	0.50	0.12	2.15	3.85	6.68	4.20	0.23	0.41	0.21	0.85
IL18	1.36	4.48	1.67	2.38	0.86	0.02	0.98	0.05	1.76	4.26	1.90	1.83	0.39	0.66	0.89	0.35
IL6	11.64	8.06	1.86	2.19	0.04	0.00	0.46	0.03	15.52	4.65	3.43	3.42	0.03	0.42	0.58	0.74
ITGA10	2.11	11.12	1.53	2.26	0.10	0.02	0.68	0.11	2.24	3.06	3.12	1.95	0.46	0.79	0.34	0.43
JKAMP	2.11	1.94	2.04	1.51	0.50	0.93	0.90	0.35	3.36	2.52	2.03	2.54	0.80	0.67	0.31	0.83
Jun	2.21	1.76	2.36	1.84	0.41	0.23	0.54	0.20	0.82	1.85	0.90	1.68	0.01	0.72	0.40	0.16
MAFF	8.53	4.00	2.05	1.83	0.10	0.09	0.83	0.52	4.96	1.48	2.35	0.99	0.82	0.43	0.95	0.08
MAP3K12	5.28	4.77	2.33	2.28	0.06	0.01	0.09	0.01	2.23	3.28	4.72	2.76	0.41	0.36	0.04	0.86
MDF1	6.27	5.30	6.65	1.70	0.50	0.75	0.00	0.30	15.73	9.37	15.29	9.08	0.30	0.16	0.00	0.21
MET	1.49	1.83	1.31	1.76	0.45	0.24	0.78	0.24	0.83	1.28	1.25	1.04	0.03	0.44	0.76	0.05
MSTN	0.62	1.04	1.52	2.22	0.17	0.75	0.78	0.34	0.48	1.33	0.74	1.22	0.00	0.18	0.17	0.02
MYC	20.34	5.76	2.32	1.66	0.04	0.01	0.86	0.29	10.92	5.27	2.55	2.50	0.82	0.54	0.65	0.25
MYF5	1.84	3.57	1.48	1.35	0.05	0.04	0.75	0.21	2.36	2.69	1.83	1.63	0.32	0.71	0.71	0.17
MYF6	1.39	2.01	1.80	2.51	0.95	0.52	0.89	0.01	4.98	3.70	1.58	3.26	0.63	0.62	0.75	0.51
MYH1	0.64	1.81	0.35	1.81	0.14	0.24	0.01	0.19	4.43	5.10	4.35	5.75	0.94	0.75	0.97	0.84
MYH2	0.94	1.19	1.51	1.35	0.23	0.52	0.91	0.57	2.11	2.14	1.41	2.13	0.33	0.16	0.54	0.27
MYH3	1.39	1.69	4.56	10.13	0.54	0.04	0.46	0.12	3.13	3.32	5.62	3.83	0.88	0.03	0.05	0.99
MYH4	1.59	1.72	1.81	2.29	0.87	0.43	0.86	0.56	2.56	2.85	2.68	4.06	0.73	0.90	0.44	0.82
MYH7	1.62	1.77	1.76	1.33	0.86	0.49	0.51	0.51	0.75	0.99	1.02	0.89	0.01	0.17	0.26	0.02
MYOG	2.00	3.04	1.42	4.57	0.79	0.13	0.73	0.06	1.94	2.44	1.24	1.69	0.44	0.74	0.50	0.13
NEDD4	0.80	1.05	1.60	1.87	0.30	0.36	0.96	0.29	1.92	1.77	0.93	1.64	0.65	0.25	0.18	0.29
NFKb1	2.84	2.03	1.11	1.57	0.09	0.07	0.46	0.16	1.85	1.95	1.61	1.23	0.22	0.85	0.96	0.08
NFKb2	4.79	3.51	1.98	2.39	0.01	0.17	0.69	0.05	5.43	4.42	2.89	2.33	0.14	0.19	0.10	0.44
PAX3	0.90	1.48	1.38	1.88	0.06	0.25	0.26	0.39	2.71	4.24	1.54	2.68	0.73	0.26	0.86	0.32
PAX7	1.51	2.23	1.38	1.47	0.92	0.14	0.54	0.08	1.38	2.67	1.87	2.19	0.01	0.66	0.41	0.31
PDK2	0.91	1.01	0.98	0.79	0.14	0.69	0.59	0.95	1.43	1.47	1.21	1.49	0.10	0.08	0.26	0.08
PDK4	4.15	2.03	1.61	2.07	0.21	0.36	0.69	0.45	6.13	1.34	2.30	0.91	0.49	0.23	0.72	0.08
PDP1	0.86	1.26	1.44	1.53	0.74	0.59	0.79	0.19	0.49	1.02	2.17	0.82	0.01	0.17	0.96	0.05
PFK1	11.44	3.90	1.78	3.93	0.06	0.01	0.39	0.11	3.28	3.79	4.09	2.94	0.70	0.13	0.06	0.21
PIK3R1	3.56	1.74	1.27	2.14	0.20	0.16	0.91	0.20	0.90	1.54	1.51	0.97	0.04	0.73	0.98	0.08
PPARGC1A	0.95	1.16	1.42	1.06	0.32	0.56	0.48	0.87	1.73	2.65	1.67	2.18	0.05	0.81	0.69	0.47

(*Continued*)

Table 3. (Continued)

Gene name	PLA								NSAID							
	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d	24 h	7 d	28 d	84 d
	Fold change from mean baseline				*P*-value				Fold change from mean baseline				*P*-value			
PRKAA1	2.50	2.27	2.02	2.03	0.16	0.53	0.80	0.11	2.27	3.16	2.31	2.70	0.19	0.58	0.17	0.96
PSMA1	1.70	1.62	1.19	1.53	0.31	0.24	0.63	0.31	1.39	1.43	1.40	1.10	0.06	0.53	0.88	0.02
PTGS1	3.35	3.63	1.79	1.10	0.05	0.00	0.55	0.18	1.94	3.23	3.92	2.86	0.13	0.19	0.05	0.64
PTGS2	2.10	8.77	1.39	1.57	0.06	0.13	0.55	0.76	8.39	5.42	6.52	8.01	0.32	0.13	0.10	0.07
PTK2	4.80	2.98	1.54	1.75	0.12	0.09	0.98	0.30	1.44	1.44	2.02	1.12	0.16	0.57	0.81	0.08
Rabb15	16.95	5.45	1.99	2.67	0.04	0.00	0.80	0.12	20.05	4.07	5.76	2.79	0.14	0.35	0.19	0.78
RPS6	2.16	1.85	1.84	1.30	0.08	0.34	0.79	0.17	3.61	2.05	1.87	1.90	0.92	0.41	0.40	0.13
RPS6KA	2.29	1.93	1.20	1.01	0.16	0.08	0.94	0.28	2.00	1.72	1.53	1.31	0.49	0.51	0.88	0.01
RRPTOR	2.12	1.67	1.32	1.29	0.37	0.87	0.45	0.21	2.09	2.40	1.62	2.11	0.09	0.46	0.58	0.25
RRAD	4.40	2.91	1.53	2.00	0.25	0.07	0.89	0.69	5.86	4.55	1.79	2.06	0.17	0.78	0.97	0.89
SCD	6.41	6.54	5.49	3.18	0.15	0.35	0.24	0.07	9.46	5.03	12.12	13.12	0.73	0.16	0.12	0.02
SIRT1	1.58	1.41	1.61	1.16	0.76	0.70	0.65	0.42	3.01	2.18	1.69	2.35	0.61	0.12	0.74	0.61
SREBF2	2.11	1.80	1.54	1.21	0.21	0.72	0.84	0.30	2.93	2.56	2.29	2.35	0.75	0.87	0.12	0.84
SRF	2.67	1.53	1.16	1.16	0.07	0.19	0.43	0.27	1.51	1.42	1.13	1.15	0.05	0.27	0.56	0.01
TGF1b	3.48	8.43	1.97	1.75	0.04	0.00	0.36	0.04	1.74	3.86	3.57	2.24	0.17	0.30	0.15	0.56
TNC	51.38	58.53	14.32	2.51	0.00	0.01	0.13	0.15	23.82	6.81	36.50	4.42	0.01	0.01	0.00	0.39
TNF	2.44	4.48	2.84	2.00	0.28	0.10	0.19	0.37	10.17	8.45	8.01	4.33	0.01	0.04	0.00	0.09
TRIM63	2.25	1.81	1.39	2.44	0.70	0.65	0.99	0.17	2.20	0.69	0.59	0.51	0.39	0.10	0.19	0.01
TSC2	2.35	2.47	1.36	1.62	0.23	0.10	0.70	0.34	0.96	1.43	1.71	1.14	0.05	0.53	0.88	0.08
Tubb2A	3.66	3.26	3.42	1.38	0.07	0.04	0.32	0.32	2.53	2.40	1.89	2.68	0.37	0.92	0.50	0.80
Tubb	4.57	4.11	1.91	1.22	0.04	0.03	0.09	0.29	4.79	4.43	6.39	3.63	0.04	0.11	0.01	0.38
TXNIP	1.04	1.40	1.66	1.17	0.90	0.80	0.79	0.60	2.50	2.60	1.34	2.93	0.38	0.52	0.45	0.69
VEGFA	1.22	1.14	1.25	0.74	0.67	0.68	0.70	0.89	1.15	1.24	1.46	1.26	0.03	0.19	0.94	0.06
YWZHA	2.45	2.42	1.70	1.38	0.07	0.12	0.84	0.46	7.28	4.25	3.37	3.38	0.03	0.09	0.00	0.33
ZFP36	7.11	2.83	2.14	1.66	0.12	0.44	0.74	0.27	6.03	2.86	1.99	2.37	0.48	0.85	0.72	0.47

Note: Values are expressed as fold change relative to the mean baseline.

PLA group at 84 days. Although the degree of change of mRNA abundance from baseline at 28 days was sufficient to pass the threshold for this cellular function to be deemed altered (Fig. 4), the number of genes associated with the function that changed was insufficient to pass the threshold for a reliable network prediction at this time point. At 84 days, however (Fig. 5*A*), four mRNAs were identified as being altered in abundance relative to baseline, of which vascular endothelial growth factor A (VEGFA), AKT1 and PPARGC1A were downregulated, and MYOG was upregulated. Based on these collective changes in mRNA abundance IPA predicted an inhibition of formation of muscle.

In the NSAID group sufficient numbers of genes were differentially regulated at 28 and 84 days for IPA network predictions to be made for the same cellular function, 'skeletal and muscular development and function', as 20 mRNAs were identified as being altered in abundance relative to baseline at 28 days (Fig. 5*B*). The most upregulated mRNAs from baseline were TNF, IGF1, IL-6 and prostaglandin-endoperoxide synthase 2 (PTGS2), whereas TRIM63 was the most downregulated transcript. Based on these collective changes IPA predicted formation of muscle to be activated with high confidence and differentiation of muscle to be inhibited with less confidence. At 84 days (Fig. 5*C*) 13 mRNAs were identified

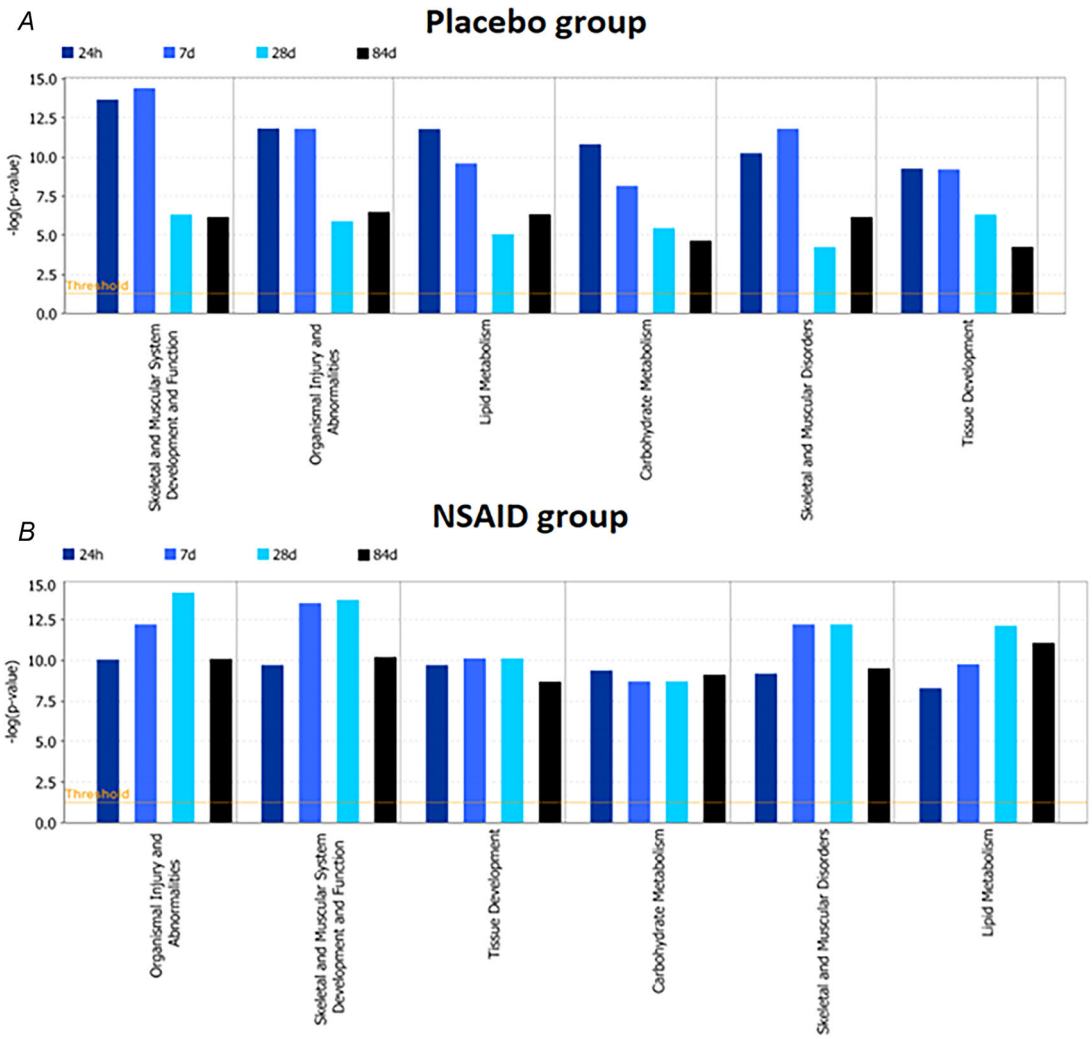

Figure 4. Altered cellular functions
Cellular functions identified using Ingenuity Pathway Analysis (IPA) as being altered from baseline (n = 8) in vastus lateralis muscle in (*A*) the groups consuming placebo (n = 8) and (*B*) diclofenac (n = 9) at 24 h, 7 days, 28 days and 84 days based on mRNA expression data generated using the low-density microarray cards. The x-axis displays cellular functions most affected by training, whereas the y-axis displays the –log of the P-value. The P-value associated with each cellular function is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance. The –log of the P-value was calculated using a right-tailed Fisher's exact test ($P < 0.05$). The threshold line corresponds to a P-value of 0.05.

as being altered in abundance relative to baseline. Similar to 28 days the most upregulated mRNAs were IGF1, PTGS2 and TNF, whereas TRIM63 was the only down-regulated transcript. Based on the collective changes IPA predicted formation of muscle activation with high confidence.

Muscle protein expression. Mature TNF-α protein expression in the PLA group was less relative to BL at 24 h ($P<0.001$) (Table 4). Mature TNF-α protein expression was also less in the NSAID group relative to BL at 7 days ($P<0.001$) and 28 days ($P<0.001$). However there was no difference in expression levels of muscle inflammatory proteins between groups at any timepoint (Table 2).

Discussion

This is the first study to quantify the time course of effects of chronic NSAID ingestion on resistance training-induced increase of muscle CSA, muscle volume

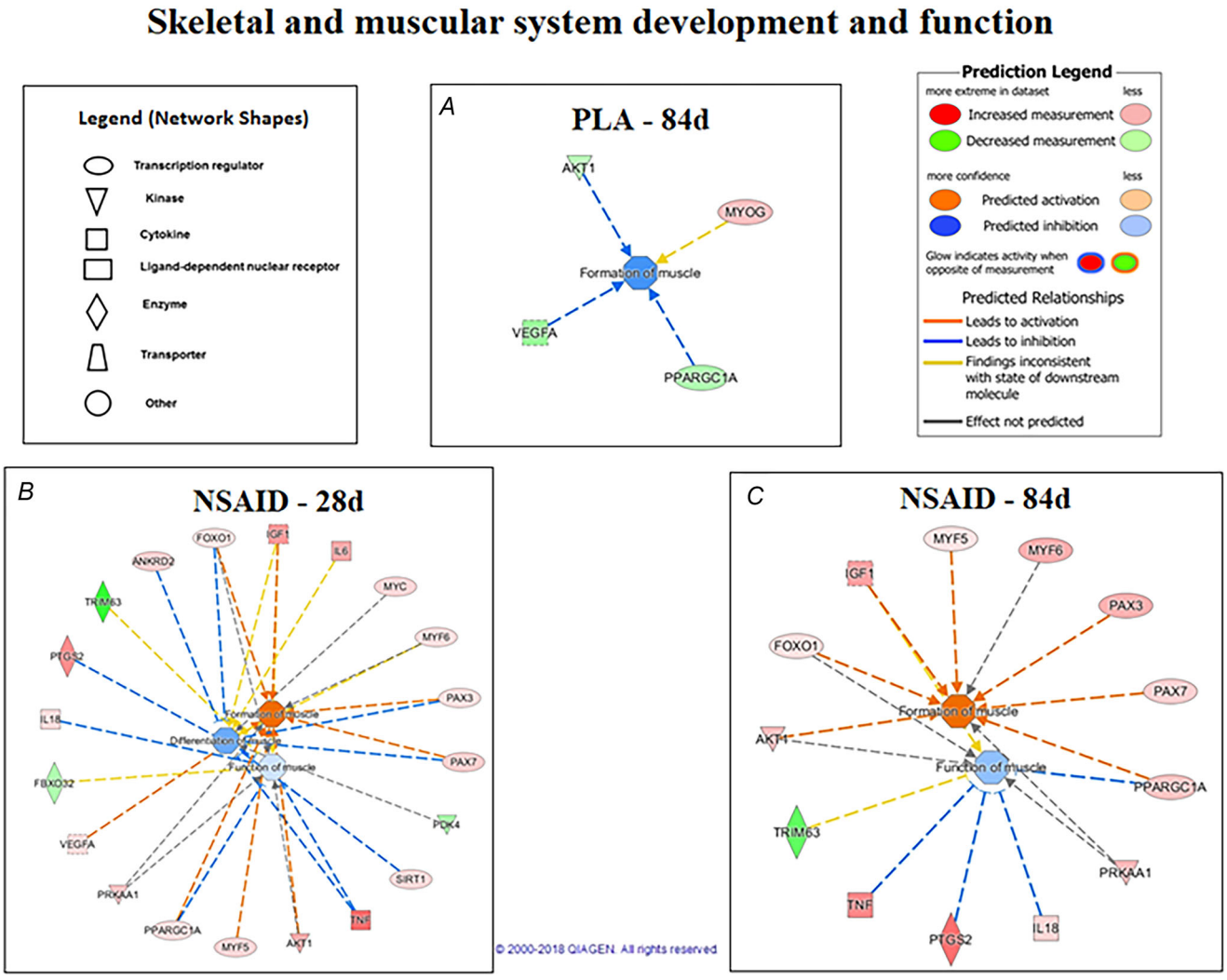

Figure 5. Muscle gene networks
Differentially regulated muscle mRNAs (outer ring) associated with skeletal and muscular system development and function in the groups consuming placebo at 84 days (*A*) and diclofenac (NSAID) at (*B*) 28 days and (C) 84 days compared to baseline, and the cellular events predicted using Ingenuity Pathway Analysis to result from the collective changes in mRNA abundance. Abbreviations: AKT1, serine threonine kinase 1; ANKRD2, ankyrin repeat domain 2; FBXO32, F-box only protein 32; FOXO1, forkhead box protein O 1; IGF1, insulin-like growth factor 1; IL-6, interleukin 6; IL-18, interleukin 18; MYC, MYC proto-oncogene; MYF5, myogenic factor 5; MYF6, myogenic factor 6; MYOG, myogenin; PAX3, paired box gene 3; PAX7, paired box gene 7; PDK4, pyruvate dehydrogenase kinase 4; PTGS2, prostaglandin-endoperoxide synthase 2; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRKAA1, protein kinase AMP-activated catalytic subunit alpha 1; SIRT1, sirtuin 1; TNF, tumour necrosis factor; TRIM 3, tripartite motif containing 63; VEGFA, vascular endothelial growth factor A.

Table 4. Expression levels of inflammatory proteins, such as interleukin-6 (IL-6), tumour necrosis factor alpha precursor (TNF-α precursor) and mature tumour necrosis factor alpha (TNF-α mature), in vastus lateralis muscle in the groups consuming placebo (PLA; *n* = 8) and diclofenac (NSAID; *n* = 9) at 24 h, 7 days, 28 days and 84 days

Protein	Treatment	24 h	7 d	28 d	84 d
IL-6	PLA	0.65 ± 0.59	0.95 ± 1.02	1.38 ± 1.24	0.91 ± 0.86
	NSAID	0.86 ± 0.63	0.62 ± 0.45	0.50 ± 0.25	0.84 ± 0.97
TNF-α precursor	PLA	0.96 ± 0.45	0.99 ± 0.47	1.08 ± 0.78	1.19 ± 0.63
	NSAID	0.73 ± 0.40	0.90 ± 0.28	0.97 ± 0.45	0.99 ± 0.77
TNF-α mature	PLA	0.28 ± 0.20***	0.43 ± 0.58	0.51 ± 0.8	0.53 ± 0.79
	NSAID	0.75 ± 1.18	0.40 ± 0.11***	0.41 ± 0.09***	1.35 ± 1.68

Note: Values are expressed as fold change relative to the mean baseline ± SD. Baseline set at 1. ***$P < 0.001$ compared to baseline.

and strength; and the abundance of mRNAs linked to regulation of muscle mass and muscle metabolism in young, trained participants. The major finding was that 84 days of NSAID ingestion increased muscle CSA and volume gains in response to maximal intensity concentric exercise training above that seen with placebo intervention. This augmentation of muscle hypertrophy occurred predominantly between days 28 and 84 of training. In parallel with this, gene networks associated with several cellular functions linked to regulation of muscle mass and metabolism were found to be more altered with NSAID ingestion than with placebo at 28 days and 84 days of training. Notably strength gains were not increased in the NSAID group above placebo at any time-point. Furthermore, both training work output per week and total work output over the duration of the protocol were not different between groups.

Few studies have investigated the impact of chronic NSAID administration on resistance exercise-induced muscular adaptations in young adults. Krentz et al. (2008) showed that in healthy and young participants performing 60 combined concentric-eccentric elbow flexor contractions, 5×/week over 6 weeks, concurrent daily ingestion of the NSAID ibuprofen had no effect on muscle hypertrophy or strength over placebo ingestion. However the short duration of training of this trial may be a limitation, given that the present study detected the difference in training-induced increase in muscle CSA, and volume between NSAID and PLA groups occurred between 28 and 84 days of training (Fig. 3). In the PLA group neither muscle CSA nor muscle volume increased during this period, which was unexpected, but could be a consequence of low statistical power. Nevertheless increases in muscle mass (Ogasawara et al., 2013) and postexercise MPS (Brook et al., 2015) have been reported to plateau with chronic resistance training. However from 28 to 84 days of training in the NSAID group muscle CSA and volume increased by ∼ 120% further, which is a novel finding. In agreement with the current findings Trappe et al. (2011) reported greater muscle volume gains (∼ 47%) in older volunteers consuming ibuprofen (1200 mg/day) compared to a placebo during 12 weeks of progressive resistance training.

When considered in the context of published literature on NSAID administration and exercise-induced muscle hypertrophy, the results of the current study differ from comparable studies in young and healthy individuals. This could be at least partly due to differences in the duration of resistance exercise training (e.g. 6 *vs.* 12 weeks), the type of contractions performed (e.g. concentric *vs.* plyometric), the limb studied (arm *vs.* leg) and the intensity and total number of contractions executed per bout of training. For example volunteers in the current study performed a total of 5400 maximal, isokinetic, concentric, knee extension contractions over 12 weeks as opposed to a total of 1800 biceps contractions (concentric at 70% 1 RM and eccentric at 100% 1 RM) over 6 weeks in the study by Krentz et al. (2008), where no impact of NSAIDs on muscle hypertrophy was reported. This suggests that a high dose of exercise stimulus may be required to achieve NSAID-induced augmentation of resistance exercise-mediated muscle hypertrophy in young, healthy individuals. Additionally differences in NSAID selectivity and administered dose could help position the findings of the current study within the broader published literature. For example, as highlighted earlier, diclofenac has a strong preference for inhibition of COX-2 isoenzymes, whereas ibuprofen is a more potent COX-1 inhibitor, which may therefore help explain why ibuprofen administration had little impact on muscle hypertrophy over 6 weeks of resistance exercise training in the study of Krentz et al. (2008). More recently Roberts and colleagues have investigated the impact of consuming a single prophylactic dose of three NSAIDs varying in COX-1/COX-2 selectivity on post-exercise muscle signalling (Roberts, Geddis et al. 2024) and functional performance (Roberts, Sczuroski et al. 2024) in healthy adult volunteers in double-blind, cross-over design studies. The latter study revealed celecoxib, a preferential COX-2 inhibitor, attenuated decreases in

maximal voluntary contraction force 4 h postexercise, compared to ibuprofen and flurbiprofen, suggesting there may be COX selectivity with respect to postexercise functional recovery.

The lack of between-group differences in muscle proinflammatory protein expression levels in the present study, in the face of clear between group differences in the hypertrophic response, point to exercise-induced low-grade muscle inflammation having little impact on regulation of muscle mass under the current experimental conditions, as opposed to scenarios of overt inflammation, as in the critically ill, in whom change in muscle IL-6 and TNF-α mRNA expression has been shown to be far greater than in controls (6.5-fold; $P<0.001$ and 2-fold; $P<0.01$, respectively) (Constantin et al., 2011) and associated with muscle wasting (Puthucheary et al., 2013). However it is acknowledged that muscle biopsy samples were not obtained in the immediate postexercise period, and because of this any acute postexercise-induced inflammatory/NSAID drug interaction will have been missed.

In the current study isometric strength of the knee extensors was increased by training from baseline to 84 days by $\sim$ 40% to 50% on average. However there were no differences between groups at any time point, demonstrating that the greater muscle hypertrophy in the NSAID group did not translate to increased strength. Importantly this is not an isolated finding in studies examining the impact of pharmacological interventions on muscle mass and functional gains. For example a number of effective anabolic pharmacological agents have been shown to result in similar disconnect in strength gains, including selective androgen receptor modulators (SARMs) (Negro-Vilar, 1999) and myostatin inhibitors (Rooks et al., 2017). Only supra-physiological doses of testosterone have been shown to increase muscle mass and strength above placebo (Bhasin et al., 1996). Moreover anabolic steroids were abandoned as muscle size and strength-promoting therapies due to myriad adverse drug reactions at the higher doses required to improve performance (Darden, 1983).

As alluded to earlier it seems to have been accepted that the analgesic effect of chronic NSAID use enables greater training workloads to be performed, thereby indirectly enhancing muscle strength and size gains. However in the present study neither total work output per week of training nor work done over the entire protocol differed between PLA and NSAID (Fig. 2), which detracts from this widely held view.

Thus far no study has identified the molecular response to combined chronic NSAID administration and resistance training in a multiple time point, longitudinal study design. In the present study, based on collective changes in mRNA abundance from baseline, IPA predicted a number of cellular functions to be altered in both intervention groups during training (Fig. 4). However the magnitude of alteration in these functions was $\sim$2-fold greater in the NSAID group at 28 days and 84 days and importantly paralleled the greater increase in muscle CSA and volume gains in these volunteers. Indeed for the cellular function 'skeletal and muscular system development and function', IPA predicted the inhibition of muscle formation in the PLA group at 84 days (Fig. 5*A*), which contrasted with the high confidence prediction of activation of muscle formation in the NSAID group at 28 days (Fig. 5*B*) and 84 days (Fig. 5*C*). Furthermore these IPA-based predictions in the NSAID group were based on mRNA changes that included increased expression of myogenic transcription factors, MYF5 and MYF6, and transcripts linked to satellite cell activation and proliferation, PAX3 and PAX7. Collectively these findings also corroborate that the greater hypertrophy in the NSAID group was unlikely to be attributable to MRI artefacts – in particular, an increase in muscle water content – which might have been reasonably suspected given NSAIDs are known to cause water retention, possibly through their actions on prostaglandins (Villa et al., 1997).

In summary this study found that chronic administration of NSAIDs in young, recreationally active male volunteers over 84 days of training comprising unilateral maximal concentric knee extensions increased muscle CSA and volume gains above a group consuming placebo, and that this occurred predominantly from 28 days to 84 days of training. In parallel with this greater alterations in gene networks associated with a number of cellular functions linked to regulation of muscle mass and metabolism were detected in the NSAID group relative to the placebo. The augmentation of resistance training-induced muscle CSA and volume gains during combined NSAID ingestion, and resistance training was not accompanied by improvements in training work output above resistance training alone as the protocol progressed or by a greater increase in muscle strength. It would therefore be incautious to recommend chronic NSAID ingestion alongside resistance training to augment strength gains, but they may be advantageous to the restoration of muscle mass loss following injury and trauma.

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Additional information

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

None declared.

Author contributions

J.M., T.T: acquisition, analysis or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work. D.C.-T.: conception or design of the work; acquisition, analysis or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work. M.F.: acquisition, analysis or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work. D.A.: acquisition, analysis or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work. P.G.: conception or design of the work; acquisition, analysis or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council [grant number BB/I020713/1], Medical Research Council [grant number MR/P021220/1] and Versus Arthritis [grant reference 21 595].

Previously published data

Data pertaining to the PLA intervention of the current study have previously been published (Mallinson et al., 2020), but the aims and objectives of the study were in no way overlapping with the current NSAID intervention study.

Acknowledgements

The authors would like to thank the staff of the David Greenfield Human Physiology Unit and all volunteers who participated in this research.

Keywords

hypertrophy, muscle strength, NSAID, resistance exercise, skeletal muscle

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Peer Review History