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Ibuprofen treatment blunts early translational signaling responses in human skeletal muscle following resistance exercise

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Markworth JF, Vella LD, Figueiredo VC, Cameron-Smith D. Ibuprofen treatment blunts early translational signaling responses in human skeletal muscle following resistance exercise. *J Appl Physiol* 117: 20–28, 2014. First published May 15, 2014; doi:10.1152/japplphysiol.01299.2013.—Cyclooxygenase-1 and -2 pathway-derived prostaglandins (PGs) have been implicated in adaptive muscle responses to exercise, but the role of PGs in contraction-induced muscle signaling has not been determined. We investigated the effect of inhibition of cyclooxygenase-1 and -2 activities with the nonsteroidal anti-inflammatory drug ibuprofen on human muscle signaling responses to resistance exercise. Subjects orally ingested 1,200 mg ibuprofen (or placebo control) in three 400-mg doses administered ~30 min before and ~6 h and ~12 h following a bout of unaccustomed resistance exercise (80% one repetition maximum). Muscle biopsies were obtained at rest (preexercise), immediately postexercise (0 h), 3 h postexercise, and at 24 h of recovery. In the placebo (PLA) group, phosphorylation of ERK1/2 (Thr202/Tyr204), ribosomal protein S6 kinase (RSK, Ser380), mitogen-activated kinase 1 (Mnk1, Thr197/202), and p70S6 kinase (p70S6K, Thr421/Ser424) increased at both 0 and 3 h postexercise, with delayed elevation of phospho (p)-p70S6K (Thr389) and p-rpS6 (Ser235/S36 and Ser240/244) at 3 h postexercise. Only p-ERK1/2 (Thr202/Tyr204) remained significantly elevated in the 24-h postexercise biopsy. Ibuprofen treatment prevented sustained elevation of MEK-ERK signaling at 3 h (p-ERK1/2, p-RSK, p-Mnk1, p-p70S6K Thr421/Ser424) and 24 h (p-ERK1/2) postexercise, and this was associated with suppressed phosphorylation of ribosomal protein S6 (Ser235/236 and Ser240/244). Early contraction-induced p-Akt (Ser473) and p-p70S6K (Thr389) were not influenced by ibuprofen, but p-p70S6K (Thr389) remained elevated 24 h postexercise only in those receiving ibuprofen treatment. Early muscle signaling responses to resistance exercise are, in part, ibuprofen sensitive, suggesting that PGs are important signaling molecules during early postexercise recovery.

NSAID; mTOR; ERK; protein synthesis; inflammation; prostaglandins

RESISTANCE EXERCISE ELEVATES the rate of skeletal myofiber protein synthesis during postexercise recovery, a response that is thought to contribute to long-term increases in muscle mass and strength with chronic resistance training (1). Exercise-induced muscle injury and the associated inflammatory response have been proposed as one potential mechanistic link between resistance exercise, muscle protein synthesis, and skeletal muscle hypertrophy (43, 44, 58). The cyclooxygenase (COX) enzymes, COX-1 and -2, catalyse the biosynthesis of the prostaglandins (PGs; PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2) and thromboxanes (TX; TXA_2); arachidonic acid derived autocrine/paracrine inflammatory lipid mediators. Blockade of the PG synthesis pathway with COX-1 and -2 inhibiting nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to have deleterious effects on rodent myofiber regeneration and adaptive growth (2, 3, 31, 46, 49). Although confirmative studies showing a negative effect of NSAID treatment on long-term human muscle adaptation are currently lacking, treatment with nonselective NSAIDs has been reported to block the acute human muscle protein synthesis (55, 57) and satellite cell proliferative (29) responses to a single bout of eccentric exercise. On this basis, it has been suggested that PGs may be important signaling molecules in skeletal muscle tissue during postexercise recovery (56).

Muscle protein synthesis is tightly regulated at the level of mRNA translation initiation, with the mammalian-target of rapamycin (mTOR) pathway playing a central role in the control of translational efficiency (17, 54, 60). mTOR acts via phosphorylating downstream effectors, including p70S6 kinase (p70S6K) and eukaryotic initiation factor (eIF) 4E binding protein. Exercise-induced p70S6K phosphorylation in human muscle significantly correlates with the magnitude of acute elevation in protein synthetic rate during postexercise recovery (4, 14, 21) and ultimately gains in muscle mass with chronic exercise training (27, 53). Downstream phosphorylation of ribosomal protein S6 (rpS6) by p70S6K (and potentially other upstream kinases) plays an important role in the control of muscle cell size (28, 41, 42). The extracellular receptor kinase (ERK) branch of the mitogen-activated protein kinase (MAPK) pathway is another signaling mechanism that appears to be important in the control of muscle protein synthesis (12, 19, 37, 52) and myofiber size/muscle mass (15, 37, 47, 48). ERK1/2 signaling regulates the translational apparatus directly via phosphorylation of translation initiation and elongation factors, including eIF4B (22, 45), eIF4E (13), and eukaryotic elongation factor 2 kinase (61). Additionally, the downstream ERK target, p90 rpS6 kinase (RSK), can cross talk with mTOR pathway components by phosphorylating signaling proteins both upstream (tuberous sclerosis complex 2) (23, 38, 39) and downstream (p70S6K Ser424/Thr421 and rpS6 Ser235/236) of mTOR (18, 35, 40). ERK1/2 is rapidly phosphorylated in human skeletal muscle postexercise, suggesting that the MEK-ERK pathway plays a synergistic role in the control of muscle protein synthesis during postexercise recovery (20).

Few studies have explored the potential links between COX-1 and -2 pathway-derived PGs and muscle cell signaling. We have recently investigated the effect of resistance exercise on PG biosynthesis and the role of PGs as signaling molecules in muscle cells in vitro. First, we showed that that $PGF_{2\alpha}$ stimulates mTOR signaling in cultured C_2C_{12} myotubes via a MEK-ERK-dependent pathway (25). Second, we reported that heighted endogenous PG synthesis by cultured muscle cells in

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response to provision of free arachidonic acid substrate induces a robust hypertrophic myotube phenotype via a COX-2-dependent pathway (24). Last, we found that PG levels are increased in human peripheral blood during the early hours (0–3 h postexercise) of exercise recovery, and this response can be blocked by preexercise (Pre) ingestion of the NSAID ibuprofen (26). In the present study, we investigated the effect of inhibition of PG biosynthesis during recovery via ibuprofen treatment on resistance exercise-induced translational signaling responses in human muscle. We hypothesized that blockade of PG biosynthesis with ibuprofen treatment may interfere with intracellular signal transduction pathways thought to be involved in muscle protein synthesis following resistance exercise.

MATERIALS AND METHODS

Participants. Sixteen healthy young male subjects (age 23 ± 0.7 yr, height 189 ± 0.02 cm, body mass 88 ± 3.1 kg) [detailed subject characteristics previously reported (26)] volunteered and provided informed, written consent to participate in the study, after the nature, purpose, and risks of the study were explained. Exclusion criteria deemed that participants must have abstained from lower body resistance exercise training for a period ≥6 mo and had no history of chronic anti-inflammatory drug use. Current use of medications or a previous history of a diagnosed condition or illness that would endanger the participants during strenuous exercise and/or anti-inflammatory drug treatment also excluded the participants from participation. All procedures involved in this study were formally approved by the Deakin University Human Research Ethics Committee.

Testing and familiarization. Participants underwent a familiarization session 1 wk before the experimental trial when repetition maximum strength testing was also performed to determine experimental exercise load [80% of one repetition maximum (1 RM)]. The maximal weight that subjects could lift for three to six repetitions (3–6 RM) on the barbell squat, leg press, and leg extension exercises was determined, and participants' 1 RM was estimated using the Brzycki equation [1 RM = 100 × load rep/(102.78 − 2.78 × reps completed)]. Participants were instructed to abstain from any vigorous physical activity in the following week before the experimental trial day.

Experimental protocol. The evening before the exercise trial, participants were provided with a standardized meal [carbohydrate (CHO) 57%, fat 22%, protein 21%] to be consumed before 10 PM. Participants arrived the following morning (7:00 AM *day 1*) in the fasted state. A summary of the experimental design is presented in Fig. 1. During ~30 min of supine rest, a 20-gauge cannula was inserted in the anticubital vein to allow venous blood sampling. Participants were randomly allocated to either the NSAID (1,200 mg/day oral ibuprofen) (n = 8) or the PLA group (gelatin capsules identical in appearance containing powdered sugar in place of ibuprofen) (n = 8). Participants ingested the first dose of ibuprofen (400 mg) on arriving at the laboratory on *day 1*, immediately before undergoing baseline blood/muscle sampling. Participants then rested supine following collection of resting muscle biopsy and blood samples for ~10–15 min, at which time the exercise protocol commenced. Following a 10-min warm up consisting of light cycling on a bicycle ergometer and one low-resistance warm-up set on each exercise, participants performed three sets each of 8–10 repetitions of bilateral barbell smith rack squat, 45° leg press, and seated knee extension at 80% 1 RM. Participants rested for 1 min between each exercise and 3 min between sets. As reported by our laboratory previously, the average time that elapsed between initial Pre NSAID dose ingestion and immediate postexercise blood and muscle biopsy sampling averaged 48.69 ± 3.35 min (26). Our laboratory has published previous intramuscular signaling work using this resistance exercise protocol (59) and reported in a recent companion paper that

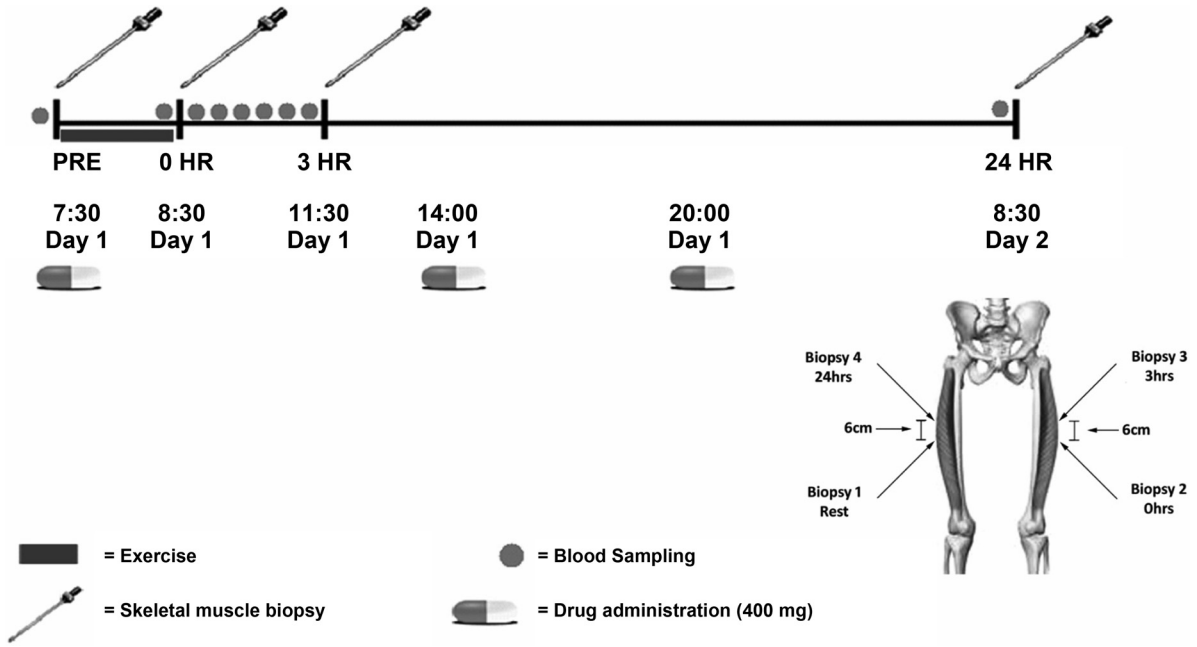

Fig. 1. Experimental study design. Participants ingested the first 400-mg dose of ibuprofen or placebo at ~7:30 AM on *day 1*, immediately before undergoing a baseline muscle biopsy sample from their dominant leg. The mean ± SE time elapsed between preexercise (Pre) ibuprofen ingestion and immediate postexercise muscle and blood sampling was 48.69 ± 3.35 min (26). Immediate postexercise biopsy samples were taken within 1–2 min of completion of the last contraction from subject's nondominant limb. Further postexercise muscle biopsies were obtained from subject's nondominant leg and dominant leg at 3 h recovery [3 h and 48.69 ± 3.35 min following the 1st nonsteroidal anti-inflammatory drugs (NSAIDs) dose] and ~24 h postexercise, respectively (~12 h following the 3rd and final NSAID dose). Sequential biopsies on the same limb were taken through a second incision 6 cm proximal to the previous biopsy site. Peak circulating ibuprofen concentrations were achieved 101.25 ± 17.87 min postexercise (149.94 ± 21.22 min post-NSAID ingestion) (26).

PG biosynthesis was elevated during the early recovery period (0–3 h postexercise) in these same participants (26).

Following completion of the exercise bout, subjects rested supine throughout a 3-h recovery period during which postexercise muscle biopsy and blood samples were collected (Fig. 1). Participants were then provided with a pre-prepared standardized meal to be consumed at the laboratory (CHO 71%, fat 13%, protein 16%). Additional snacks and a standardized evening meal (CHO 64%, fat 27%, protein 18%) were provided for subjects to consume throughout the remainder of the trial day. Participants were instructed not to consume any additional food or drink. Two additional 400-mg ibuprofen doses or placebo pills were provided, which subjects were instructed to ingest at 2:00 PM (~6 h postexercise) and 8:00 PM (~12 h postexercise) on the evening of the trial day (1,200 mg total daily dose) (Fig. 1). The participants returned to the laboratory the following morning (8:00–9:00 AM), following an overnight fast, for 24 h postexercise measurements (Fig. 1).

Muscle biopsies. Skeletal muscle biopsy samples were collected from the *vastus lateralis* musculature under local anesthesia (xylocaine 1%, 1–2 ml per biopsy site) by percutaneous needle biopsy technique modified to include manual suction. Biopsies were taken before exercise (Pre), immediately postexercise (0 h), and following 3 h, and 24 h of recovery (Fig. 1). *Biopsies 1* and *4* were taken from participant's dominant leg, and *biopsies 2* and *3* from the contralateral (nondominant leg). The immediate postexercise biopsy was collected within 1–2 min of completion of the last muscle contraction. Sequential biopsies from the same limb were obtained through a separate incision 6 cm proximal to the previous biopsy site. All skeletal muscle biopsy samples were immediately frozen in liquid nitrogen (<1 min from sample removal).

Tissue processing and immunoblotting. Tissue was homogenized in ice-cold RIPA lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA supplemented with protease and phosphatase inhibitors, including 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM Na_3VO_4, and 1 mM NaF), and homogenates were agitated for 1 h at 4°C. Homogenates were centrifuged at 13,000 *g* at 4°C for 15 min, and the supernatant was collected and stored at −80°C until further analysis. Total protein content of the muscle homogenate was determined with a BCA-protein kit (Pierce). Aliquots of 50-μg total protein were prepared, suspended in Laemmli buffer, boiled, and subjected to SDS-PAGE. The four muscle biopsy samples from each participant were always run in contiguous lanes on the same gel, and each gel always contained samples from both PLA and ibuprofen (IBU) group subjects. Proteins were transferred to a polyvinylidene difluoride membrane, and blocked in 5% BSA/Tris buffer saline/0.1% Tween 20 (TBST) for 2 h at room temperature, followed by overnight incubation at 4°C with gentle agitation with primary antibodies {phospho (p)-Akt (Ser473), total Akt, p-p70S6K (Thr389), p-p70S6K (Thr421/Ser424), total p70S6K, p-ERK1/2 (Thr202/Tyr204), total ERK1/2, p-RSK (Ser380), p-Mnk1 (Thr197 and 202), total Mnk1, p-rpS6 (Ser240/244), p-rpS6 (Ser235/236), total rpS6, p-STAT-3 [signal transducer and activator of transcription-3 (STAT-3)] (Tyr705), and p-p38MAPK (Thr380/Tyr382); all obtained from Cell Signaling Technologies}. Membranes were then washed for 30 min with TBST and probed with horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Following 30-min further washing in TBST, antibody binding was visualized using Western Lighting enhanced chemiluminescence reagent (PerkinElmer Life Sciences). Signals were captured using a Kodak Digital Science Image Station 440CF (Eastman Kodak), and densitometry band analysis was undertaken with Kodak Molecular Imaging Software (version 4.0.5, Eastman Kodak). Protein phosphorylation level was normalized by stripping and reprobing phosphorylated protein probed membranes with antibodies specific to the matching total protein or to a close correspondent total protein from the same pathway, if a matching total/phosphor-protein antibody pair was unavailable (p-RSK Ser380). Phospho-protein results were normalized to total protein abundance.

Data analysis. Western blot densitometry data for each protein of interest was expressed relative to each subject's Pre biopsy sample. Fold change from Pre data were compared across time points and between the PLA and IBU groups using a two-way ANOVA with repeated measured for time (SigmaPlot version 12.0). Following statistically significant main or interaction effects, Student-Newman-Keuls post hoc tests were used to determine the significance of pairwise comparisons between individual time points and groups, with $P \leq 0.05$ considered statistically significant. All data are presented as the mean ± SE.

RESULTS

Ibuprofen kinetics and effect on PG biosynthesis. As previously reported by our laboratory (26), the ibuprofen dosing protocol used was effective in elevating serum ibuprofen concentrations in the blood samples drawn immediately postexercise and maintained these levels throughout 3 h of postexercise recovery. Serum ibuprofen was no longer elevated 24 h postexercise [~12 h following the third and final ibuprofen dose (26)]. Peak elevation in circulating COX-1 and -2-derived TXs and PGs was observed between immediately post- (TXB_2), 1–2 h post- (PGD_2, PGE_2, $PGF_{2\alpha}$), and 24 h (6-keto-$PGF_{1\alpha}$) postexercise, and these responses were significantly suppressed in those receiving ibuprofen treatment (26). In addition to the expected effect of ibuprofen on COX-1 and -2-derived PGs and TXs, several other classes of bioactive lipid mediators were also significantly altered during postexercise recovery in those receiving ibuprofen treatment, and these findings have been discussed in detail elsewhere (26).

MEK-ERK pathway. Phosphorylation of ERK1/2 at Thr202/Tyr204 increased significantly from baseline levels in the PLA group, but not the IBU group, immediately postexercise (PLA, $P < 0.001$; IBU, $P > 0.05$ vs. Pre) (Fig. 2*A*) and remained elevated only in the PLA group at both 3 h and 24 h of recovery (PLA, $P < 0.01$; IBU, $P > 0.05$) (Fig. 2*A*). Phosphorylation of the downstream ERK1/2 target, RSK at Ser380 increased in both the PLA ($P < 0.001$) and IBU groups ($P < 0.01$) immediately postexercise, with significantly higher phosphorylation in the PLA group compared with the IBU group at this time point (PLA vs. IBU, $P < 0.05$) (Fig. 2*B*). At 3 h postexercise, p-RSK Ser380 remained significantly elevated above baseline in the PLA group only ($P < 0.01$) and also tended to remain significantly blunted in the IBU group compared with the PLA group (PLA vs. IBU, $P = 0.08$) (Fig. 2*B*). At 24 h of recovery, p-RSK Ser380 had returned to baseline levels in both groups, with no significant difference between groups. Phosphorylation of Mnk, an additional downstream target of ERK, at the Thr197/202 residues of Mnk1 (and the corresponding residues of Mnk2 with which the antibody used cross reacts) increased in the PLA group ($P < 0.001$), but not the IBU group ($P > 0.05$) immediately postexercise (Fig. 2*C*). At 3 h postexercise, p-MNK remained significantly elevated above baseline in the PLA group ($P < 0.01$), but did not differ from baseline within the IBU group. Furthermore, Mnk1/2 phosphorylation was found to be significantly suppressed (PLA vs. IBU, $P < 0.01$ at 0 h postexercise), or displayed a trend toward significantly lower phosphorylation (PLA vs. IBU, $P = 0.09$, 3 h postexercise) in the IBU group compared with the PLA group at the same respective time point (Fig. 2*C*).

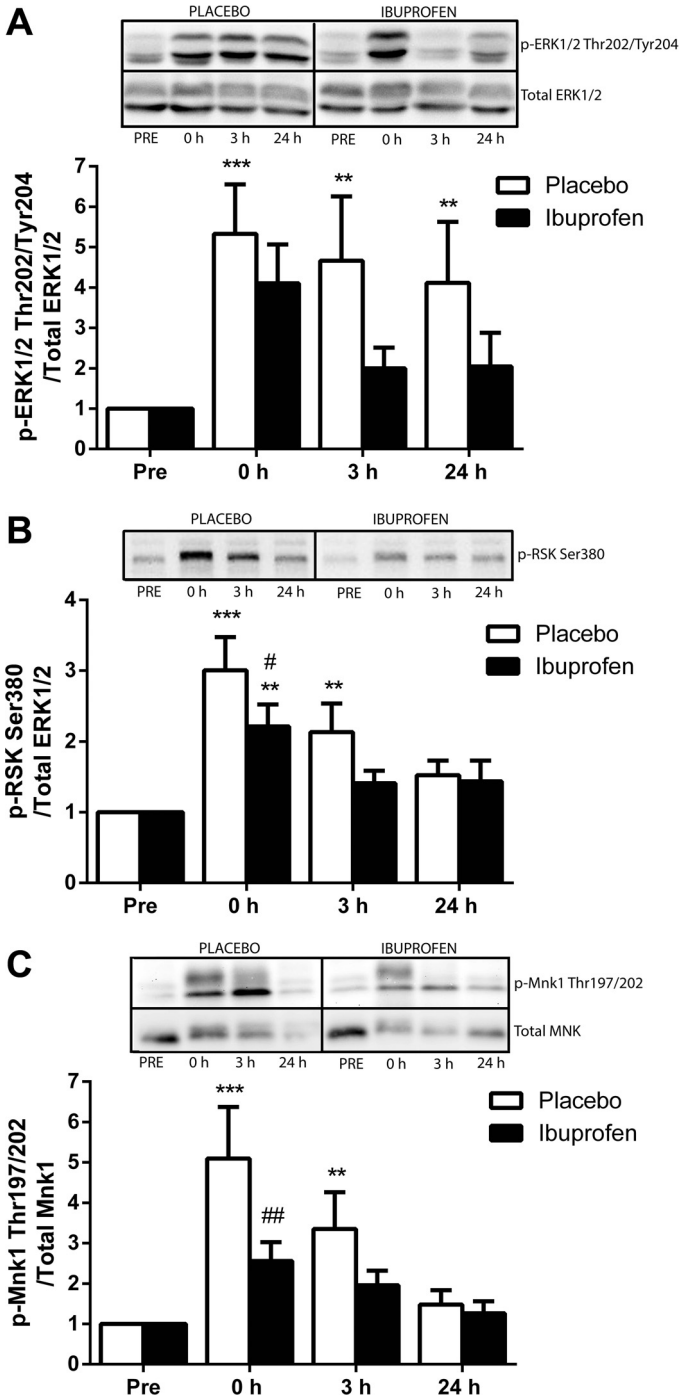

Fig. 2. Ibuprofen treatment blocks sustained elevation of muscle MEK-ERK signaling during postexercise recovery. Phosphorylation (p) of ERK1/2 at Thr202/Tyr204 (*A*), ribosomal protein S6 kinase (RSK) at Ser380 (*B*), and Mnk1 at Thr197/202 (*C*) in human muscle during postexercise recovery for subjects receiving placebo (open bars) or ibuprofen (solid bars) is shown. Values are means ± SE of $n = 8$ per group. ***$P < 0.001$, **$P < 0.01$ vs. Pre. ##$P < 0.01$, #$P < 0.05$ for ibuprofen vs. placebo group at the same time point.

Akt. Phosphorylation of Akt at Ser473 displayed a trend toward a modest and transient decrease (~20% vs. Pre, nonsignificant) immediately postexercise, followed by subsequent significant elevation from this trough 3 h postexercise in both groups ($P < 0.05$ vs. 0 h postexercise) (Fig. 3*A*). At 24 h post-resistance exercise, p-Akt Ser473 remained elevated vs. the 0-h postexercise time point in both groups. Despite these differences between postexercise time points, no significant changes from baseline were achieved at any postexercise time point in either group. Additionally, no significant differences between the PLA and IBU groups were observed in Akt Ser473 phosphorylation at any time point (Fig. 3*A*).

p70S6K. The phosphorylation of p70S6K at Thr389 was unchanged immediately postexercise in either group, but increased significantly the above baseline levels at 3 h postexercise in both the PLA and IBU groups (both $P < 0.001$ vs. respective Pre levels) (Fig. 3*B*), with no significant difference between groups. At 24 h postexercise, p-p70S6K Thr389 was no longer significantly elevated above baseline levels in the PLA group, but did, however, remain elevated in the IBU group ($P < 0.05$ vs. Pre) (Fig. 3*B*).

Phosphorylation of p70S6K at Ser424/Thr421 increased immediately postexercise in both the PLA group ($P < 0.001$) and the IBU group ($P < 0.001$), with no significant difference between groups (Fig. 4*A*). p-p70S6K Ser424/Thr421 remained

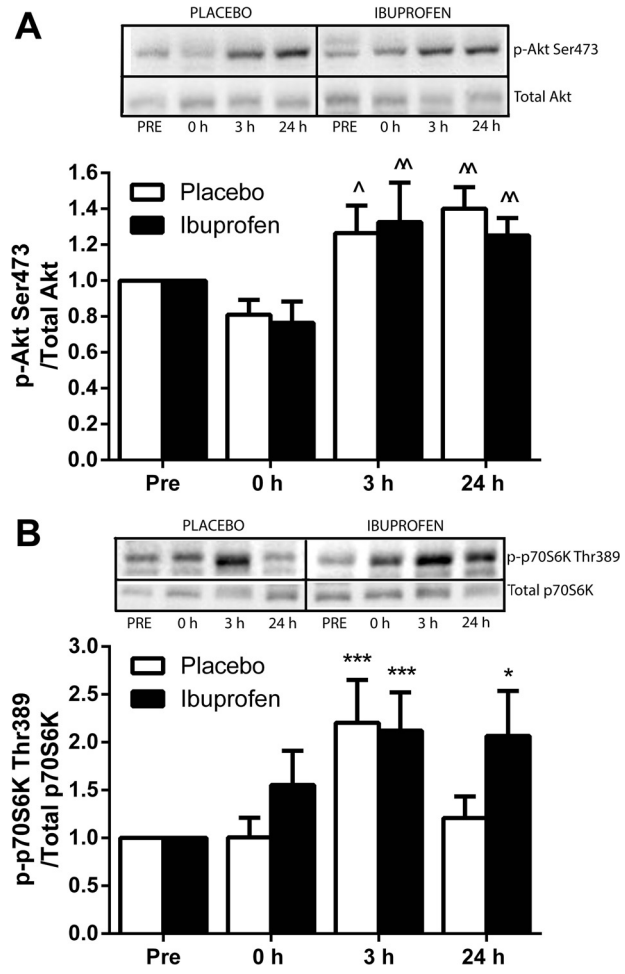

Fig. 3. No effect of ibuprofen on Akt/mammalian target of rapamycin (mTOR) signaling during early recovery, but sustained and/or delayed phosphorylation of p70S6 kinase (p70S6K) Thr389 in the ibuprofen group at 24 h postexercise. Phosphorylation of Akt at Ser473 (*A*) and p70S6K at Thr389 (*B*) in human muscle during postexercise recovery for subjects receiving placebo (open bars) or ibuprofen (solid bars) is shown. Values are means ± SE of $n = 8$ per group. ^^$P < 0.01$, ^$P < 0.05$ vs. immediately postexercise time point. ***$P < 0.001$, *$P < 0.01$ vs. Pre.

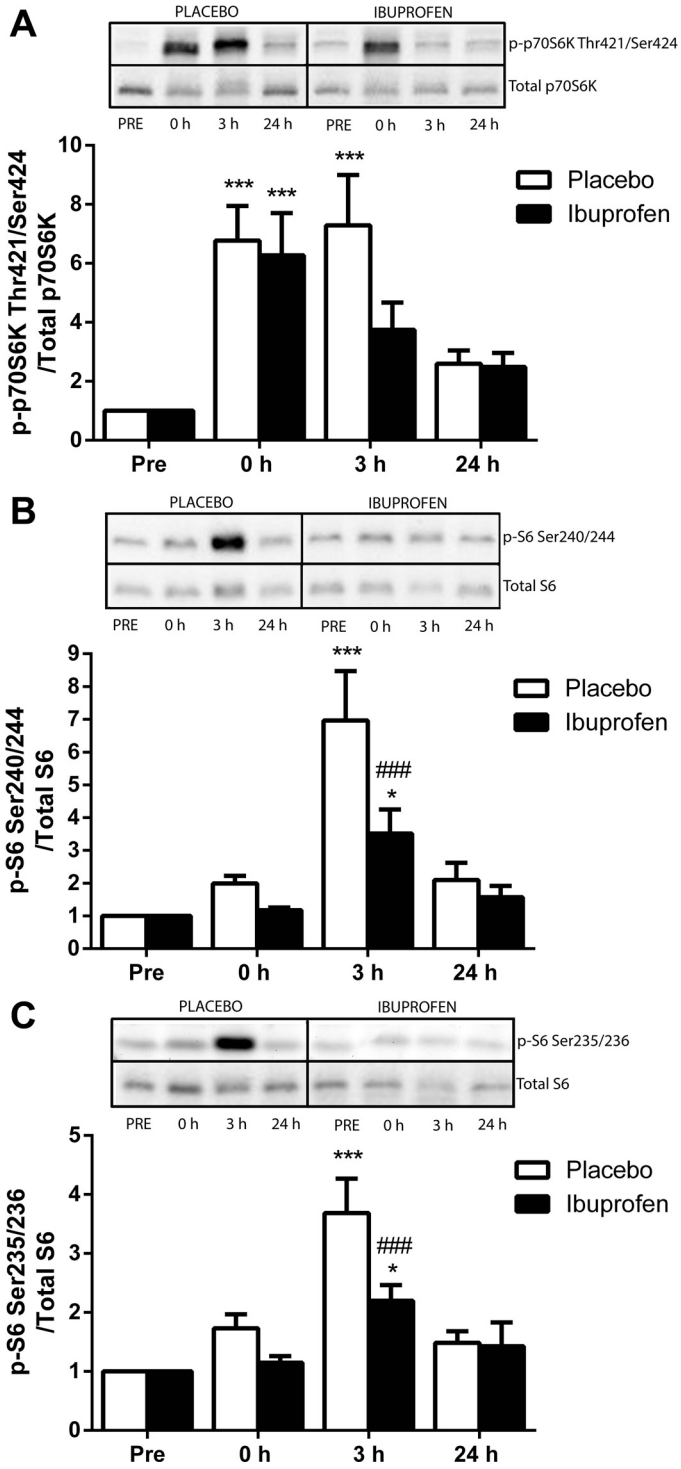

Fig. 4. Suppressed downstream muscle translational signaling responses in those receiving ibuprofen treatments. Phosphorylation of p70S6K at Thr421/Ser424 (*A*), rpS6 at Ser240/244 (*B*), and rpS6 at Ser235/236 (*C*) in human muscle during postexercise recovery for subjects receiving placebo (open bars) or ibuprofen (solid bars) is shown. Values are means ± SE of $n = 8$ per group. ***$P < 0.001$, *$P < 0.05$ vs. Pre. ###$P < 0.001$ for placebo vs. ibuprofen groups at the same time point.

elevated 3 h postexercise in the PLA group only (PLA, $P < 0.001$ vs. Pre) (Fig. 4*A*). Twenty-four hours postexercise, p-p70S6K Ser424/Thr421 was no longer significantly elevated from baseline for either group.

rpS6. Immediately postexercise, phosphorylation of rpS6 at both Ser235/235 and Ser240/244 residues did not differ significantly from baseline (Fig. 4, *B* and *C*). Three hours postexercise, phosphorylation of rpS6 at both Ser240/244 and Ser235/236 increased significantly in both the PLA ($P < 0.001$) and IBU ($P < 0.05$) groups, but these responses were found to both be significantly suppressed in the IBU group compared with the PLA group (PLA vs. IBU 3 h postexercise, p-rpS6 Ser240/244, $P < 0.001$; p-rpS6 Ser235/236, $P < 0.01$) (Fig. 4, *B* and *C*). Twenty-four hours postexercise, phosphorylation of rpS6 at both Ser240/244 and Ser235/236 had returned to Pre levels with no significant difference between groups.

p38 MAPK and STAT-3. Phosphorylation of p38MAPK Thr180/Try182 increased immediately postexercise in both the PLA group ($P < 0.01$) and IBU group ($P < 0.01$) (Fig. 5*A*). At 3 h and 24 h postexercise, p-p38MAPK Thr180/Try182 was no longer elevated above baseline in either group. Phosphorylation of STAT-3 Tyr705 was unchanged immediately postexercise, but increased at 3 h in both the PLA group ($P < 0.05$) and the IBU group ($P < 0.01$), with no difference between groups observed (Fig. 5*B*). Twenty-four hours postexercise, p-STAT-3 Tyr705 was no longer elevated above baseline for either group.

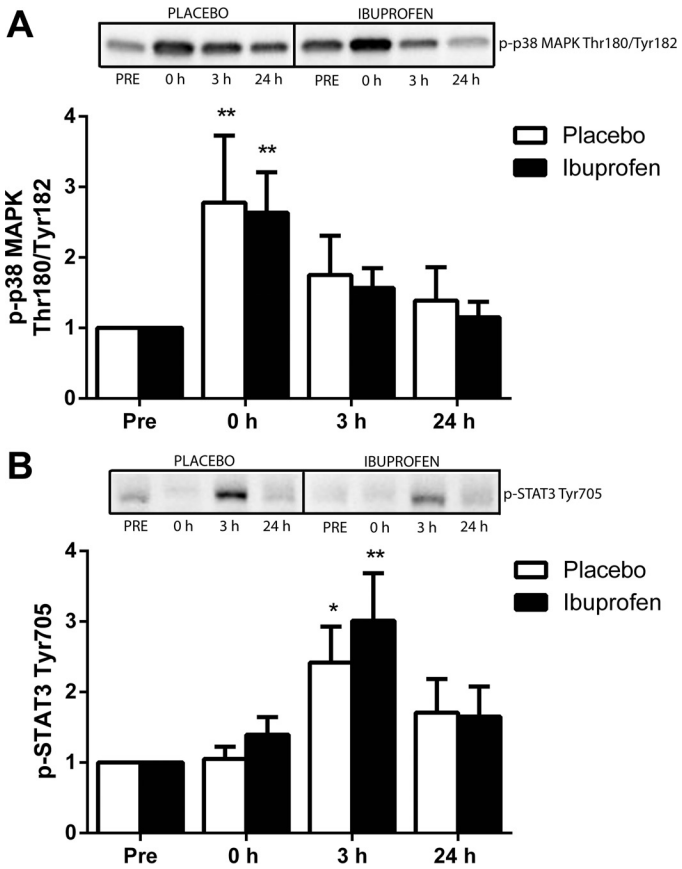

Fig. 5. No effect of ibuprofen treatment on contraction-induced p38 and signal transducer and activator of transcription-3 (STAT-3) signaling responses. Phosphorylation of p38 MAPK Thr180/Tyr182 (*A*) and STAT-3 at Tyr705 (*B*) in human muscle during postexercise recovery for subjects receiving placebo (open bars) or ibuprofen (solid bars) is shown. Values are means ± SE of $n = 8$ per group. **$P < 0.01$, *$P < 0.05$ vs. Pre.

DISCUSSION

The present study was undertaken to determine the effect of ibuprofen treatment on translational signaling responses in human muscle following a bout of unaccustomed resistance exercise. As previously reported by our laboratory, the ibuprofen dosing protocol used was successful in suppressing the normal elevation in PG biosynthesis during early postexercise recovery in these subjects (26). In the present study, we showed that sustained phosphorylation of ERK1/2, RSK, Mnk, and p70S6K Ser424/Thr421 at 3 h of postexercise recovery in human muscle was prevented by ibuprofen treatment, while rpS6 phosphorylation at both Ser235/236 and Ser240/244 was significantly suppressed. Ibuprofen, however, had no apparent negative influence on the phosphorylation of Akt Ser474 or p70S6K Thr389. These findings suggest that the early muscle signaling response to resistance exercise is, in part, ibuprofen sensitive, and highlight a role for COX-1 and -2-derived PGs as important signaling molecules in human muscle during postexercise recovery. Furthermore, suppressed downstream phosphorylation of rpS6 in those receiving ibuprofen, despite intact upstream Akt Ser473 and p70S6K Thr389 responses, may be indicative of contribution of MEK-ERK pathway cross talk to the exercise-induced rpS6 response in human muscle.

Given the apparent role of mTOR signaling in exercise-induced muscle protein synthesis (9, 10), and recent studies implicating PGs in muscle cell signaling (25), human myofiber protein turnover (55, 57), and cultured myotube hypertrophy (24), we investigated the effect of NSAID treatment on human muscle contraction-induced translational signaling responses. The traditional paradigm that resistance exercise upregulates mTOR signaling via induction of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, secondary to the systemic or local release of IGF-I, has been brought into question (36). Neither a functional skeletal muscle IGF-I receptor (50, 63), nor intact PI3K activity (16, 30, 32) appear to be required for load-induced phosphorylation of p70S6K at the mTOR-dependent Thr389 site in animal models of muscle overload. Nevertheless, consistent with some previous studies reporting elevated Akt phosphorylation in human muscle in response to resistance exercise (e.g., Refs. 5, 7, 10), we did observe a tendency toward a modest increase in the phosphorylation of Akt Ser473 at 3 and 24 h postexercise. However, elevated Akt phosphorylation was only significant compared with the 0-h postexercise time point during which p-Akt Ser473 rather tended to be modestly suppressed, as previously reported (e.g., Ref. 8). Ibuprofen treatment had no influence on the exercise-induced Akt Ser473 phosphorylation, suggesting that PGs do not appear to contribute to resistance exercise-induced Akt response. Consistently, unlike classical insulin/IGF-I signaling, $PGF_{2\alpha}$ signaling appears to have little or no effect on Akt Ser473 phosphorylation in C_2C_{12} myotubes (25). Nevertheless, treatment with $PGF_{2\alpha}$ stimulates robust phosphorylation of p70S6K at Thr389 in C_2C_{12} myotubes by a rapamycin-sensitive pathway, suggesting that PGs can regulate mTOR kinase activity under certain circumstances (25). Contraction-induced p70S6K Thr389 phosphorylation occurred to a comparable extent in the PLA and IBU groups in the present study, suggesting that contraction-induced elevation of mTOR kinase activity in human muscle during postexercise recovery does not appear to require PG biosynthesis. This finding is in agreement with a previous study, which showed that the COX-2-selective NSAID NS-398 significantly impaired the skeletal muscle hypertrophy response to rodent synergist ablation overload, despite not exerting any apparent negative effect on muscle p70S6K Thr389 phosphorylation (31).

The kinase activity of p70S6K is controlled by multiple phosphorylation events, and, while phosphorylation at Thr389 correlates best with p70S6K activity in vivo (62), phosphorylation at Ser424/Thr421 is also thought to contribute to activation of p70S6K via relief of pseudosubstrate suppression (11, 18). We found phosphorylation of p70S6K Ser424/Thr421 was rapidly increased immediately postexercise in the PLA group and exhibited sustained elevation throughout 3 h of postexercise recovery. As noted previously by other investigators (8, 51), immediate postexercise phosphorylation of p70S6K at Ser424/Thr421 temporally preceded subsequently delayed elevation in the phosphorylation at p70S6K Thr389, potentially indicative of regulation of these residues by a distinct upstream kinases. Consistently, the early muscle p70S6K Thr389 response to the rodent synergist ablation muscle overload model was recently found to be rapamycin sensitive (mTOR dependent), while phosphorylation of p70S6K at the Ser424/Thr421 residues on the other hand was rapamycin resistant (30). Both ERK and p38 MAPK activities have been shown to contribute to phosphorylation of p70S6K at Ser424/Thr421 in cultured C_2C_{12} myotubes (8) and elevated mTOR kinase activity following skeletal muscle overload in animal models was recently reported to persist in the presence of PI3K/Akt blockade due to intact phosphorylation of tuberous sclerosis complex at the purported ERK-dependent Ser664 site (30). In the present study, elevated phosphorylation of ERK1/2 and two of its downstream targets, RSK and Mnk, were associated with heightened p70S6K Ser424/Thr421 phosphorylation immediately postexercise in human muscle. In the PLA group, significant rapid induction of ERK1/2, RSK, Mnk, and p70S6K Ser424/Thr421 phosphorylation occurred immediately postexercise and were sustained above baseline levels throughout 3 h of recovery. In the IBU group, however, only phosphorylation of RSK and p70S6K Ser424/Thr421 achieved significant elevation immediately postexercise, and none of the proteins within the MEK-ERK pathway (ERK1/2, RSK, Mnk, p70S6K Ser424/Thr421) showed sustained phosphorylation following 3 h of postexercise recovery. Collectively, these findings suggest that contraction-induced MEK-ERK signaling in human muscle is at least partially ibuprofen sensitive. Interestingly, this finding appears to be consistent with previous reports of a deleterious effect of NSAIDs on the MEK-ERK signaling pathway in other cell types (33, 34).

The phosphorylation of rpS6 was unchanged immediately postexercise but significantly elevated in both groups at 3 h of recovery. Peak elevation of rpS6 phosphorylation coincided with phosphorylation of p70S6K at Thr398, consistent with the notion that p70S6K is thought to be a major upstream kinase regulating rpS6 (40). Nevertheless, despite a lack of effect of ibuprofen treatment on phosphorylation of p70S6K Thr389, we did observe significantly blunted phosphorylation of rpS6 (using two independent antibodies specific for different phosphorylation residues) in those receiving ibuprofen treatments. It is possible that contraction-induced phosphorylation of p70S6K Ser424/Thr421 by ERK/RSK may be important in contributing to mTOR-independent p70S6K activity and thus downstream

signaling to rpS6, despite intact p70S6K Thr389 phosphorylation. Alternatively, the MEK-ERK signaling pathway may directly regulate phosphorylation of rpS6 at certain residues independent of the action of p70S6K, as has been observed in other cell types previously (40). Consistent with our findings in human muscle, the early contraction-induced phosphorylation of rpS6 at both Ser240/244 and Ser235/236 in rodent skeletal muscle was recently found to be partially rapamycin resistant (30).

It is noteworthy that a lack of an effect of ibuprofen on some of the signaling responses immediately postexercise may be due to the dosing regimen employed in the present study. Subjects ingested the first 400 mg dose of ibuprofen during the Pre period, immediately before baseline muscle biopsy and blood sampling. As reported previously by our laboratory (26), the average time that elapsed between the initial Pre NSAID dose ingestion and immediate postexercise blood and muscle biopsy sampling was found to average 48.69 ± 3.35 min. Serum ibuprofen was elevated to effective circulating levels in these participants in the immediately postexercise sampled bloods, and these levels were maintained throughout the 3-h recovery sampling period (26). However, we cannot discount the possibility that some ibuprofen-sensitive signaling events may have occurred during contractions performed within the exercise bout, before effective circulating levels of ibuprofen being achieved. Such rapid contraction-induced events could have potentially contributed to some of the immediate postexercise signaling responses observed. Circulating PGs (with the exception of TXB_2) were, however, found to not be significantly elevated in these subjects until 1–2 h postexercise, consistent with the notion that the peak PG response to resistance exercise may be somewhat delayed compared with immediate contraction-induced signaling events during early postexercise recovery (26).

In summary, we found that ibuprofen inhibits MEK-ERK signaling in human muscle during postexercise recovery. Despite apparently intact phosphorylation of Akt Ser473 and p70S6K Thr389, diminished MEK-ERK signaling in ibuprofen-treated individuals was also associated with blunted delayed phosphorylation of p70S6K Ser424/Thr421 and an impaired downstream rpS6 response, implying that PGs are

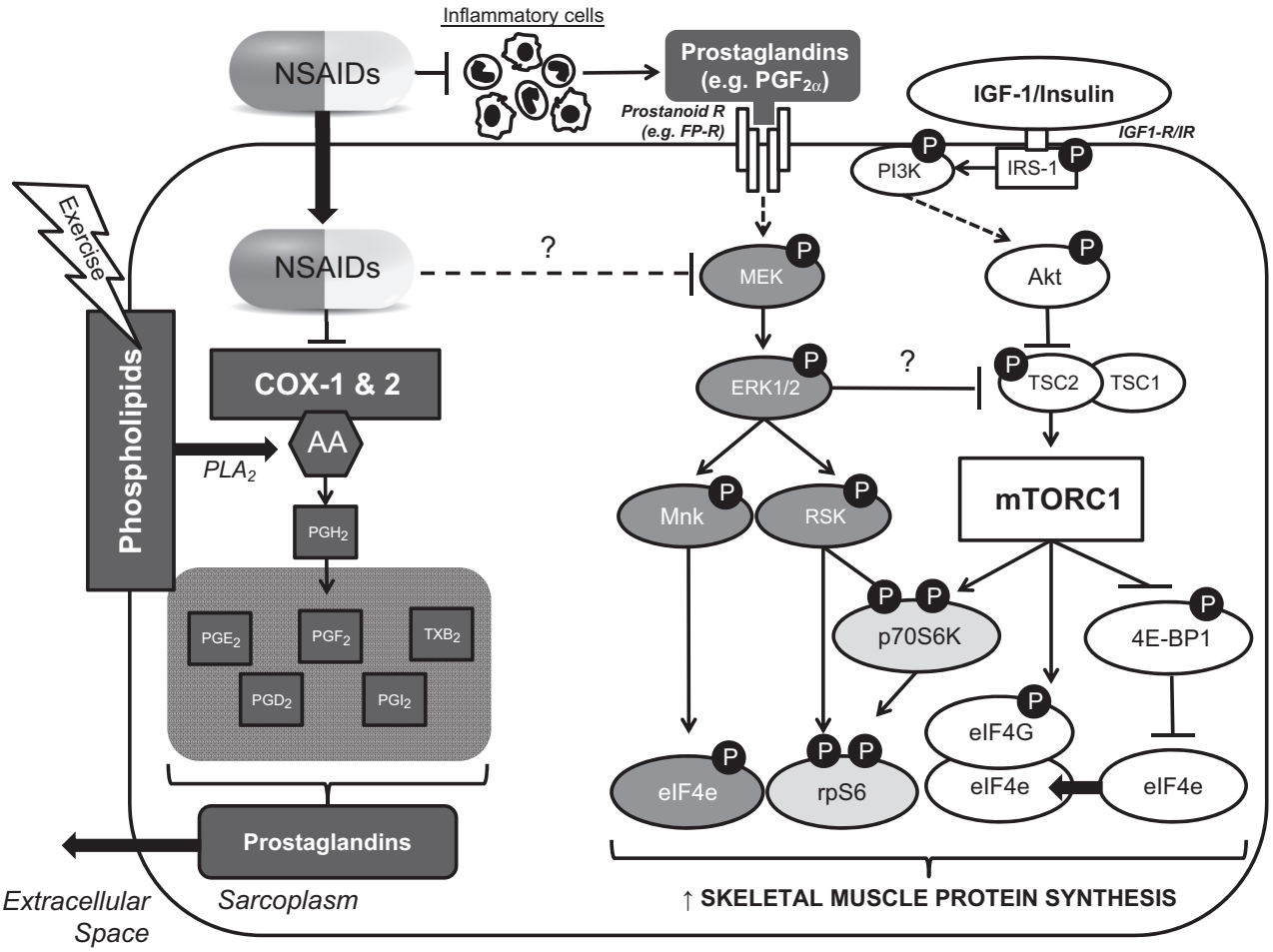

Fig. 6. Proposed pathways by which NSAID treatment may influence muscle translational signaling during postexercise recovery. In response to exercise, arachidonic acid (AA) is released from membrane phospholipids (PL) via the action of phospholipase enzymes (e.g., PLA_2). Increased free AA substrate (26) and inducible cyclooxygenase (COX)-2 expression (6) drive elevated biosynthesis of prostaglandins (PGs) during postexercise recovery (26). NSAIDs may influence exercise-induced muscle signaling by mechanisms including *1*) blockade of PG (e.g., $PGF_{2\alpha}$) autocrine/paracrine muscle signaling via cell surface receptors (e.g., FP receptor) (24, 25); *2*) diminished leukocyte trafficking blocking muscle-immune cell interactions; *3*) direct inhibitory effect on muscle cell signaling, as has been reported previously for the MEK-ERK pathway in nonmuscle cells (33, 34). Shading from darkest to none represents NSAID mechanism of action, MEK-ERK signaling pathway, MEK-ERK and Akt-mTOR pathway cross talk, and Akt-mTOR pathway, respectively. PI3K, phosphatidylinositol 3-kinase; 4E-BP1, 4E binding protein 1; eIF4, eukaryotic initiation factor 4; IRS-1, insulin receptor substrate-1.

important signaling molecules in loaded human muscle. Additionally, our findings provide evidence of cross talk between the ERK and mTOR signaling pathways in human muscle during early postexercise recovery. Figure 6 presents a schematic illustration of the potential pathways by which NSAID administration may influence contraction-induced skeletal muscle signaling responses on the basis of the results of the present and previous studies (25, 26). We conclude that resistance exercise-induced muscle signaling is, in part, ibuprofen sensitive, suggesting that PGs are important signaling molecules in human muscle during postexercise recovery.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.F.M., L.D.V., and D.C.-S. conception and design of research; J.F.M., L.D.V., V.C.F., and D.C.-S. performed experiments; J.F.M. and L.D.V. analyzed data; J.F.M., L.D.V., V.C.F., and D.C.-S. interpreted results of experiments; J.F.M. prepared figures; J.F.M. and L.D.V. drafted manuscript; J.F.M., L.D.V., V.C.F., and D.C.-S. edited and revised manuscript; J.F.M., L.D.V., V.C.F., and D.C.-S. approved final version of manuscript.

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