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Burd NA, Dickinson JM, LeMoine JK, Carroll CC, Sullivan BE, Haus JM, Jemiolo B, Trappe SW, Hughes GM, Sanders CE Jr., Trappe TA. Effect of a cyclooxygenase-2 inhibitor on postexercise muscle protein synthesis in humans. *Am J Physiol Endocrinol Metab* 298: E354–E361, 2010. First published November 24, 2009; doi:10.1152/ajpendo.00423.2009.—Nonselective blockade of the cyclooxygenase (COX) enzymes in skeletal muscle eliminates the normal increase in muscle protein synthesis following resistance exercise. The current study tested the hypothesis that this COX-mediated increase in postexercise muscle protein synthesis is regulated specifically by the COX-2 isoform. Sixteen males (23 ± 1 yr) were randomly assigned to one of two groups that received three doses of either a selective COX-2 inhibitor (celecoxib; 200 mg/dose, 600 mg total) or a placebo in double-blind fashion during the 24 h following a single bout of knee extensor resistance exercise. At rest and 24 h postexercise, skeletal muscle protein fractional synthesis rate (FSR) was measured using a primed constant infusion of [$^2H_5$]phenylalanine coupled with muscle biopsies of the vastus lateralis, and measurements were made of mRNA and protein expression of COX-1 and COX-2. Mixed muscle protein FSR in response to exercise ($P < 0.05$) was not suppressed by the COX-2 inhibitor (0.056 ± 0.004 to 0.108 ± 0.014%/h) compared with placebo (0.074 ± 0.004 to 0.091 ± 0.005%/h), nor was there any difference ($P > 0.05$) between the placebo and COX-2 inhibitor postexercise when controlling for resting FSR. The COX-2 inhibitor did not influence COX-1 mRNA, COX-1 protein, or COX-2 protein levels, whereas it did increase ($P < 0.05$) COX-2 mRNA (3.0 ± 0.9-fold) compared with placebo (1.3 ± 0.3-fold). It appears that the elimination of the postexercise muscle protein synthesis response by nonselective COX inhibitors is not solely due to COX-2 isoform blockade. Furthermore, the current data suggest that the COX-1 enzyme is likely the main isoform responsible for the COX-mediated increase in muscle protein synthesis following resistance exercise in humans.

celecoxib; [$^2H_5$]phenylalanine; resistance exercise

RESISTANCE EXERCISE HAS BEEN SHOWN to elevate muscle protein synthesis for up to 48 h in humans (29, 40). It is generally understood that, when resistance exercise is performed chronically, the accumulation of these acute increases in muscle protein synthesis is the underlying basis of muscle hypertrophy. Although the metabolic and molecular events that control muscle protein synthesis have received a lot of attention in recent years (18, 46, 66), these complex processes are not yet completely understood.

Prostaglandin (PG)$F_{2\alpha}$, a lipid compound synthesized in skeletal muscle by the cyclooxygenase (COX) enzyme, has been shown to be a potent regulator of muscle protein synthesis (23, 37, 45, 47, 61–63). For example, data from our laboratory have demonstrated that blocking the COX production of $PGF_{2\alpha}$ with ingested over-the-counter doses of ibuprofen (1,200 mg/day) or acetaminophen (4,000 mg/day) following resistance exercise abolished the normal 24-h postexercise increase in skeletal muscle protein synthesis (59, 61). However, there are two well-known isoforms of COX (COX-1 and COX-2) (22, 52, 68), and it is unclear which of the COX-1 or COX-2 isoforms are blocked by these drugs in human skeletal muscle and in turn regulate the normal COX-mediated increase in $PGF_{2\alpha}$ and muscle protein synthesis. A purported third isoform, referred to as COX-3 or COX-1b (9, 41), was considered (65), but these findings, along with other evidence (8, 25, 41), have ruled out this isoform.

Studies in animals and cell culture using COX-2 selective blockade and COX-2 gene-deficient models show COX-2 dependent muscle hypertrophy following synergist ablation and following atrophy (3, 33), muscle regeneration following injury (2, 16, 49–51), and myogenesis (30, 35). These data from animals and cell culture provide intriguing evidence that the postexercise COX-mediated stimulation of muscle protein synthesis in humans may be regulated specifically through the COX-2 enzyme.

The purpose of this investigation was to expand on previous findings (58, 59, 61, 65) regarding the role of specific COX isoforms mediating muscle protein synthesis following resistance exercise in humans. The recent development of COX-2-selective inhibitors, such as celecoxib (i.e., Celebrex) (14, 17), now make it possible to selectively target this enzyme in human studies. For example, in vitro celecoxib is >375-fold more selective for COX-2 than traditional nonsteroidal anti-inflammatory drugs (14). Therefore, we measured mixed muscle protein synthesis at rest (preexercise) and 24 h following a single resistance exercise bout in two groups, one that consumed a placebo and one that consumed a COX-2 selective inhibitor. We hypothesized that mixed muscle protein synthesis would be significantly elevated after resistance exercise in the placebo group and unchanged from rest in the COX-2 inhibitor group. COX-1 and COX-2 mRNA and protein levels were also monitored in both groups before and after exercise.

MATERIALS AND METHODS

Subjects and Experimental Design

Sixteen male subjects were recruited and randomly divided into two groups of eight, a placebo group and a COX-2 inhibitor group (Table 1). All subjects were recreationally active (i.e., they did not participate in regularly structured aerobic or resistance exercise), nonobese, nonsmokers, and did not consume any prescription or nonprescription analgesic or anti-inflammatory drug(s) chronically or

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Table 1. *Subject characteristics*

	Age, yr	Height, cm	Weight, kg	1-RM Nondominant Leg, kg	1-RM Dominant Leg, kg
Placebo	23 ± 1	179 ± 2	84.7 ± 5.4	47.2 ± 2.5	47.2 ± 2.6
COX-2 inhibitor	23 ± 1	175 ± 2	78.4 ± 4.1	49.3 ± 2.6	47.9 ± 2.6

Values are means ± SE. COX, cyclooxygenase; 1-RM, 1-repetition maximum. No significant difference ($P > 0.05$) between the placebo and COX-2 inhibitor group for all measured variables.

for the duration of the study except for those administered by the investigative team. This investigation was approved by the Institutional Review Board at Ball State University, and each subject provided written, informed consent prior to participation. The experimental design is presented in Fig. 1 and described in detail below.

Dietary and Activity Control

Each subject recorded his dietary intake for 3 days prior to the resting muscle protein synthesis study, and this intake was replicated prior to the postexercise muscle protein synthesis study. On the 3rd day of each dietary intake period, evening meals were supplied in liquid form (Ensure Plus; 57% carbohydrate, 15% protein, and 28% fat) and provided 50% of the estimated daily caloric need (1.5 times the predicted resting metabolic rate) to standardize the composition, amount, and timing (i.e., duration of the fast) of the final meal consumed prior to the resting and 24-h postexercise muscle protein synthesis measurements (6, 60, 64). In addition, subjects were asked to refrain from vigorous physical activity 3 days prior to each protein synthesis study.

Muscle Protein Synthesis

The precursor-product model was used for the determination of fractional synthesis rates via the incorporation of [$^2H_5$]phenylalanine into skeletal muscle proteins at rest and 24 h after the resistance exercise bout (Fig. 1) (40, 61). For all subjects, resting muscle protein synthesis was determined from the dominant leg, and all postexercise measurements were from the nondominant leg. Upon subject arrival to the laboratory (0500–0700), an 18-gauge catheter was inserted into an antecubital vein for infusion of the stable, isotopically labeled amino acid [$^2H_5$]phenylalanine (DLM-1258-SP; Cambridge Isotopes, Andover, MA). [$^2H_5$]phenylalanine was dissolved in 0.9% saline and passed through a 0.2-μm filter and infused at 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ with a calibrated infusion pump (PHD 2000; Harvard Apparatus, Natick, MA) preceded by a priming dose of 2.0 μmol/kg. The isotope was confirmed sterile and pyrogen free prior to use. Two muscle biopsies (1) were obtained from the vastus lateralis during each infusion following local anesthetic (lidocaine HCl 1%), one at 2 h and one at 5 h of the [$^2H_5$]phenylalanine infusion, which represented the beginning and the conclusion of the protein synthesis measurement. Each biopsy was obtained through a separate incision, and the 5-h biopsy was taken proximal to the site of the 2-h biopsy. All muscle samples were cleaned of visible fat, connective tissue, and blood and immediately frozen and stored in liquid nitrogen (−190°C) until analysis.

An 18-gauge catheter was also placed in an antecubital vein of the contralateral arm for venous blood sampling. Blood samples were taken at 2.5, 3.5, and 4.5 h of the [$^2H_5$]phenylalanine infusion for the measurement of plasma tracer enrichment.

Resistance Exercise

Approximately 1 wk following the determination of resting muscle protein synthesis, each subject returned to the laboratory and completed a bout of unilateral high-intensity eccentric knee extension exercise with each leg (Cybex Eagle; Cybex, Medway, MA) (59, 61, 65). For all subjects, the nondominant leg was exercised first. The workload was set to 120% of their concentric one-repetition maximum, determined immediately prior to the exercise bout. The exercise consisted of 10 sets of 10 repetitions, with a 60-s rest between sets for each leg. The weight was lifted manually for the subject to 180°, and the subject then lowered the weight to 90°. When the weight was lowered in less than ~0.5 s, the subject completed that set, and the weight was decreased appropriately before the start of the following set.

COX-2 Inhibitor Dose and Administration

The COX-2 inhibitor (celecoxib; Celebrex) was administered in double-blind placebo controlled fashion, as we have described previously for the administration of other nonselective COX inhibitors (7, 61). The placebo and the COX-2 inhibitor drug were administered three times over a 24-h period, once at the beginning of the exercise bout, once with the evening meal (~12 h following initial pill consumption), and a final dose that was given 1 h prior to the beginning of the [$^2H_5$]phenylalanine infusion (~8.5 h following the previous pill consumption) (Fig. 1). Each dose of the COX-2 inhibitor

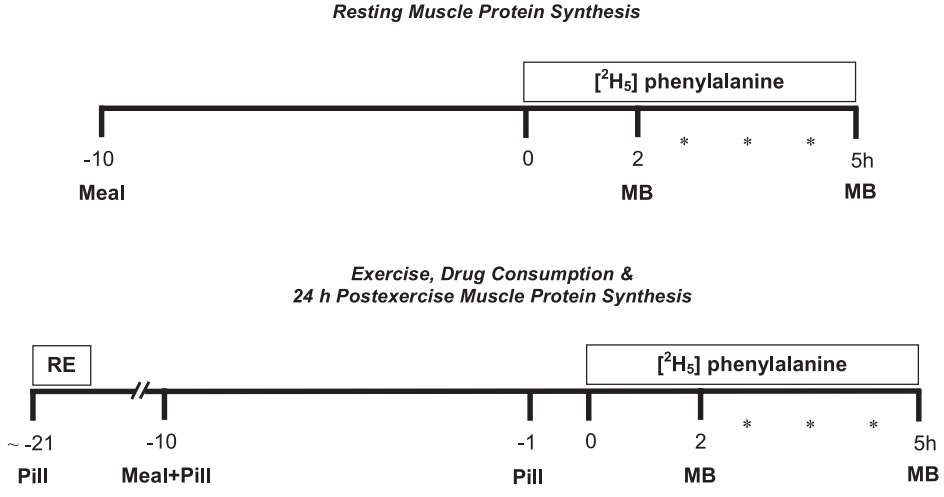

Fig. 1. Timeline of the experimental protocol. Meal, standardized meal; MB, muscle biopsy; pill, placebo or cyclooxygenase (COX)-2 inhibitor (celecoxib) consumption; RE, resistance exercise bout. *Venous blood sample. There was ~1 wk between infusion protocols.

was 200 mg, for a total of 600 mg over the 24-h period. The dosing times were chosen considering the time to reach maximal plasma concentrations (~3 h) and the half-life (~11.5 h) of the drug following oral consumption of an equal or similar single dose (14). For comparison purposes, the dosing schedule and related pharmacokinetic parameters of the nonselective COX inhibitors used in our previous study (61) were also considered. Based on typical plasma concentrations following the doses used in this and the previous study (11, 13, 14, 21, 44) and IC_{50} values (the concentration required to achieve 50% inhibition of the COX enzyme) obtained from in vitro human whole blood assays (12, 21, 32, 55), all three drugs (celecoxib, ibuprofen, and acetaminophen) should provide approximately equivalent inhibition of the COX-2 isoform in both studies. In addition, celecoxib does not provide any significant amount of COX-1 inhibition, whereas both ibuprofen and acetaminophen should block COX-1 at the doses used in each study (12, 21, 32, 55). Subjects were asked not to consume any analgesics or anti-inflammatory drugs, other than those provided by the investigative team, during the study. We assured 100% compliance with the COX-2 inhibitor and placebo consumption with observation by a member of the investigative team of all three doses for every subject.

Stable Isotope Enrichment Measurements

Sample preparation and derivatization. The rate of mixed muscle protein synthesis was determined by evaluating the muscle tissue fluid and mixed protein [$^2H_5$]phenylalanine enrichment in the muscle samples obtained from the vastus lateralis, as described previously (19). Plasma samples were also analyzed for [$^2H_5$]phenylalanine enrichment to confirm steady-state enrichment during the protein synthesis measurement period.

Each muscle sample was weighed (28.06 ± 0.55 mg) on a precision microbalance (PerkinElmer Autobalance AD-2Z; PerkinElmer, Wellesley, MA) at −35°C and then homogenized in 500 μl of ice-cold 14% perchloric acid. Muscle was homogenized on ice with a Teflon-coated pestle for 1 min and then centrifuged at 21,000 *g* for 10 min at 4°C. The supernatant was then collected, and this process was repeated two more times. All three supernatants were combined and taken as the muscle tissue fluid. The remaining pellet was washed once in purified and deionized H_2O and three more times in ethanol, with each wash followed by centrifugation at 21,000 *g* for 10 min at 4°C. The pellet, taken as the mixed protein, was then dried overnight at 50°C and then hydrolyzed in 5 ml of 6 N HCl for 24 h at 100°C. Each plasma sample was deproteinized with a 1:1 ratio of 15% sulfosalicylic acid and then centrifuged at 21,000 *g* for 10 min at 4°C.

The muscle tissue fluid, mixed muscle protein hydrolysates, and plasma samples were then washed over a cation exchange column (Dowex AG 50W-8X, 100–200 mesh, H^+ form; Bio-Rad Laboratories, Hercules, CA). Amino acids were eluted from the column with 4 N ammonium hydroxide (NH_4OH), and this eluate was collected and dried under vacuum (SC210A SpeedVac Plus; ThermoSavant, Holbrook, NY). Once dried, samples were derivatized with 100 μl of acetonitrile and *N*-methyl-*N*-*t*-butyl-dimethylsilyl-trifluoroacetamide (Pierce Chemical, Rockford, IL) at a 1:1 ratio. All samples were derivatized at 100°C, muscle tissue fluid and plasma for 10 min and mixed muscle protein for 30 min.

Gas chromatography-mass spectrometry. All samples were analyzed by gas chromatography-mass spectrometry (GC-MS; 6890N GC coupled with a 5973 inert MSD; Agilent Technologies, Wilmington, DE) in triplicate (muscle tissue fluid and mixed protein samples) or duplicate (plasma samples) using electron impact ionization and selected ion monitoring of mass-to-charge ratios 234 ($m + 0$), 235 ($m + 1$), 237 ($m + 3$), and 239 ($m + 5$), with $m + 0$ representing the lowest molecular weight of the ion. Muscle tissue fluid and plasma [$^2H_5$]phenylalanine enrichments were measured from the $m + 5$ to $m + 0$ ratio. Enrichment of the mixed protein samples was determined using the $m + 5$ to $m + 3$ ratio and a single linear standard curve from mixtures of known $m + 5$ to $m + 0$ ratios, as described previously (4, 38). Elimination of bias due to any potential concentration dependency (4, 38) was accomplished by injecting nearly identical amounts of phenylalanine (i.e., similar $m + 0$ and $m + 3$ abundances) for all samples and standards. Initial measurements were made on various amounts of phenylalanine to assure that the amount of phenylalanine injected for all samples would be below saturation levels of the detector and would produce Gaussian-shaped peaks. Coefficient of variation (CV) for duplicate injections on the GC-MS of the same plasma sample averaged 0.36 ± 0.03%. CVs for the triplicate injections of muscle tissue fluid and mixed muscle protein hydrolysate averaged 1.36 ± 0.21 and 1.89 ± 0.20%, respectively.

Calculation of muscle fractional synthesis rate. Fractional synthesis rate (FSR) was calculated as the rate of [$^2H_5$]phenylalanine incorporated into mixed muscle protein using the muscle tissue fluid phenylalanine enrichment as the precursor and the equation FSR (%/h) = $(Et_1 - Et_0)/[E_p \times (t_1 - t_0)] \times 100$, where Et_0 and Et_1 are the enrichments in the mixed protein from biopsies at $t = 2$ and 5 h (basal) and $t = 2$ and 5 h (24 h postexercise), $(t_1 - t_0)$ is the phenylalanine tracer incorporation time, and E_p is the muscle tissue fluid enrichment (average of 2 and 5 h) (19, 40, 61).

COX mRNA Measurements

Real-time RT-PCR was completed to determine the mRNA levels of the known COX isoforms and variants, COX-1 [both variant 1 (COX-1v1) and variant 2 (COX-1v2) (15, 48)], COX-2, and the intron-retaining COX-1 [also referred to as COX-3 (9)] variants (COX-$1b_1$, -$1b_2$, and -$1b_3$), as we have described previously (65).

Total RNA extraction and RNA quality check. A portion of the 2-h biopsy sample from the preexercise and postexercise stable isotope infusion protocols was used for the COX mRNA measurements. For each biopsy sample, a piece of muscle (14.26 ± 0.81 mg) previously frozen in liquid nitrogen was weighed on a precision microbalance (PerkinElmer Autobalance AD-2Z) at −35°C and placed into 400 μl of RNAlater-ICE (Ambion, Austin, TX) and stored at −20°C for ≥24 h prior to RNA extraction. Each muscle sample was removed from the RNAlater-ICE and placed in a mixture of 0.8 ml of RNA isolation reagent, TRI Reagent, and 4 μl of PolyAcryl Carrier (Molecular Research Center, Cincinnati, OH). The tissue was homogenized and total RNA extracted according to the manufacturer's protocol. The RNA pellet was dissolved in 30 μl of nuclease-free water and then stored at −80°C.

One microliter of each total RNA extract was analyzed using the RNA 6000 Nano LabChip kit on an Agilent 2100 Bioanalzyer (Agilent Technologies, Palo Alto, CA). This system reported detailed information about quantity and quality (integrity and purity) of the RNA samples. Each RNA sample was electrophoretically separated into two peaks of 18S and 28S ribosomal RNA. Data were displayed as a gel-like image and an electropherogram (24). Sample analyses were performed as described by the manufacturer. The quality of RNA was confirmed by the presence of ribosomal peaks, with no additional signals (DNA contamination or total RNA degradation) below the ribosomal bands and no shifts to lower fragments and a RNA integrity number >7 (8.7 ± 0.1).

Reverse transcription. Oligo(dT) primed first-strand cDNA was synthesized using SuperScript II RT (Invitrogen, Carlsbad, CA). This system was optimized for sensitive RT-PCR on low amounts of RNA. A first reaction mix of 12 μl for each sample, consisting of 3 μl of RNA extract (150 ng), 1 μl of 10 mM dNTP, 1 μl of oligo(dT) 12–18 (0.5 μg/μl), and 7 μl of DNase- and RNase-free water, was incubated at 65°C for 5 min and then placed on ice for 1 min. A second reaction mix of 7 μl, consisting of 4 μl of 5× first-strand buffer, 2 μl of 0.1 M DTT, and 1 μl of RNaseOUT recombinant RNase inhibitor, was then added to the first reaction mix and incubated at 42°C for 2 min. Finally, 1 μl (100 U) of SuperScript II RT was added to each tube (giving a total volume of 20 μl), incubated at 42°C for 50 min and

then 70°C for 15 min to terminate the reaction, and chilled to −4°C thereafter. Produced cDNA samples were diluted with water to a final volume of 60 μl. All thermal incubations and chilling were done in a Peltier Thermal Cycler with dual-block DNA engine (MJ Research, Waltham, MA) to provide temperature homogeneity and identical temperature ramping for all samples.

Real-time RT-PCR. Quantification of mRNA transcription (in duplicates) was performed in a 72-well Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, NSW, Australia). GAPDH was used as a housekeeping gene for internal control. The reaction mix consisted of 12.5 μl of SYBR Green JumpStart *Taq* Ready Mix (Sigma-Aldrich, St. Louis, MO), 0.5 μl of 10 μM each for forward and reverse primers, 2.5 μl of cDNA, and RNase-free water to a final volume of 25 μl. All primers used in this study were mRNA specific and designed for gene expression real-time RT-PCR analysis (Vector NTI Advance 9 software; Invitrogen) using SYBR Green chemistry. Details about primer characteristics and sequences, as well as the real-time RT-PCR parameters and amplicon melting curve analysis, have been reported previously (65).

Relative quantification of real-time RT-PCR assay. The COX gene expression before exercise and 24 h postexercise was compared using the $2^{-\Delta C_T}$ [arbitrary units (AU)] and $2^{-\Delta\Delta C_T}$ (fold change) relative quantification methods described and used by us and others previously (28, 43, 65, 67). Validation of the housekeeping gene (GAPDH) was performed to ensure that its expression was unaffected by the experimental treatments, as we have described previously (24, 43, 65, 67). The pre- and postexercise levels of GAPDH, expressed as C_T values, produced on a known amount (150 ng) of total RNA were 11.55 ± 0.11 and 11.50 ± 0.40, respectively, for the placebo group and 11.79 ± 0.36 and 11.98 ± 0.31, respectively, for the COX-2 inhibitor group. The geNorm program (http://medgen.ugent.be/~jvdesomp/genorm/download.php) was also applied to calculate the average stability expression value (M) for GAPDH and a second tested housekeeping gene, RPLPO. For both housekeeping genes, the stability expression value was 0.059 (M <1.5).

A serial dilution (1, 0.5, 0.25, 0.125, 0.062, 0.031) curve of cDNA for each gene was also amplified by real-time PCR, using gene-specific primers to evaluate reaction efficiencies. To make the dilution curve for GAPDH and the gene of interest, the cDNA from total RNA of human skeletal muscle (1 μg; Ambion) was used. The amplification calculated by the Rotor-Gene software was specific and highly efficient (efficiency = 1.12 ± 0.03, r^2 = 0.99 ± 0.00, slope = 3.19 ± 0.07).

COX Protein Measurements

A portion of the 2-h biopsy sample from the preexercise and postexercise stable isotope infusion protocols was used for the COX-1 and COX-2 protein measurements. For each biopsy sample, a piece of muscle (16.33 ± 0.75 mg) previously frozen in liquid nitrogen was weighed on a precision microbalance (PerkinElmer Autobalance AD-2Z) at −35°C. Each sample was homogenized (PowerGen 700; Fisher Scientific, Pittsburgh, PA) in 30 volumes of cold RIPA buffer (25 mM Tris·HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Pierce) with Halt Protease Inhibitor Cocktail and 5 mM EDTA (Pierce). Total protein concentration was determined (macroBCA Assay; Pierce), and aliquots of the homogenate were diluted in SDS sample buffer and heated to 95°C for 5 min. Proteins (80 μg) were separated with a 4–20% gradient gel (Pierce), using SDS-PAGE for 2 h at 75 V (Mini Protean 3 system; Bio-Rad Laboratories), and then transferred to a PVDF membrane (Immobilon-P; Millipore, Bedford, MA) for 2 h at 200 mA at 4°C. The membrane was blocked with 5% milk for 60 min and then incubated with a monoclonal COX-1 antibody diluted 1:200 (160110; Cayman Chemical, Ann Arbor, MI) in 1× Tris-buffered saline (TBS), 0.1% Tween-20 (TBS-T) with 0.5% milk, or a monoclonal COX-2 antibody diluted 1:500 (160112; Cayman Chemical) in 1× TBS-T at 4°C overnight. Blots were identified by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:7,100 (10004302; Cayman Chemical) in 1× TBS-T with 5% milk and then exposed to an enhanced chemiluminescent substrate (ChemiGlow West; Alpha Innotech, San Leandro, CA). Digital images were captured using a chemiluminescent imaging system (FluorChem SP; Alpha Innotech). Equal protein loading was verified by Ponceau S staining, and sizes of the immunodetected proteins were confirmed by molecular weight markers (Invitrogen) and a positive control COX-1 protein (60100; Cayman Chemical) or COX-2 protein (10009624; Cayman Chemical). To control for intra-assay variability, the subject's rest and postexercise samples were analyzed on the same blot, and each blot contained subjects from both experimental groups.

COX-1 considerations. The antibody used for the COX-1 measurements was not designed specifically to distinguish between the variants analyzed with the COX-1 mRNA analysis (namely COX-1 variant 1 and variant 2), and no attempt was made to distinguish which variant our measurements reflect. Only a single previous report (48) has examined specifically the protein product of COX-1v2 mRNA and suggested that this protein does not have catalytic activity or is rapidly degraded. However, their conclusions were based on examination of nonskeletal muscle tissues that showed only 5–10% mRNA expression levels of variant 2 compared with variant 1. Our data from human skeletal muscle show the mRNA expression level of variant 2 is ~50–100% higher than variant 1 [data reported here and previously (65)]. It should be noted that, although there was some nonspecific binding on the COX-1 Western blots, only one distinct band migrated with the COX-1-positive control in the skeletal muscle samples measured in the current study.

COX-2 considerations. The human COX-2 protein was first described as a 70-kDa protein (22) and has four glycosylation sites (10, 22). Some glycosylation is necessary for COX enzyme activity (34, 36, 39), and varying the glycosylation state alters the electrophoretic mobility up to ~10 kDa (27, 39), which is also influenced by the varying size of each glycosylation (31). Because of the absence of COX glycosylation details in skeletal muscle, human or animal (2, 16, 42, 54, 57, 65), the banding pattern obtained during Western blot analysis does not provide for unequivocal quantification of active COX-2. This issue has been reported on from studies in other human cell lines (27).

During extensive preliminary testing of human muscle samples, we did not detect any COX-2 band that migrated with the aforementioned recombinant human COX-2 protein (10009624; Cayman Chemical) or an ovine COX-2 electrophoresis standard protein (360120; Cayman Chemical). Detection of an amount substantially below the manufacturer's instructions for the human (0.3% of recommended) and ovine (1% of recommended) COX-2 proteins provides evidence that our sensitivity was likely not a limitation. Interestingly, purified recombinant human COX-2 generated from two different expression systems results in two different multiple banding patterns on Western blots, owing to varying glycosylation states (39). The recombinant human COX-2 protein we used, when loaded in excess, showed two distinct but much less prominent bands below the main COX-2 band. All of our muscle samples also had two detectable bands below the 70-kDa region, which appeared to be ~10 kDa smaller in size than the two COX-2 positive controls. In addition, the recombinant human COX-2 control protein was spiked into a muscle sample and further confirmed the size difference between the two lower bands and the positive control. To determine whether or not the two lower bands were indeed a nonglycosylated form of COX-2, a single experiment where a human COX-2-specific peptide (360107; Cayman Chemical) was added to the Western blot in an attempt to block COX-2 protein from binding with the monoclonal COX-2 antibody was completed. Confusingly, although the blocking successfully eliminated detection of the positive control bands, it did not eliminate the two lower bands in the muscle samples. Although the identity of these two lower bands in the muscle samples is not clear, examination showed they were not influenced by the COX-2 drug or exercise. This result is similar to our

Table 2. *Muscle tissue fluid and mixed muscle protein [$^2H_5$]phenylalanine enrichment pre- and postexercise*

	Preexercise		Postexercise	
	2 h	5 h	2 h	5 h
Muscle tissue fluid				
Placebo	0.0465 ± 0.0030	0.0502 ± 0.0025	0.0495 ± 0.0030	0.0523 ± 0.0022
COX-2 inhibitor	0.0454 ± 0.0048	0.0518 ± 0.0033	0.0558 ± 0.0028	0.0623 ± 0.0035
Mixed muscle protein				
Placebo	0.000133 ± 0.000018	0.000246 ± 0.000018	0.000457 ± 0.000026	0.000598 ± 0.000032
COX-2 inhibitor	0.000118 ± 0.000010	0.000202 ± 0.000018	0.000394 ± 0.000013	0.000586 ± 0.000032

Values are means ± SE. Numbers reflect the tracer-to-tracee ratio ($m + 5/m + 0$).

previous study (65), in which we quantified the upper of these two bands, likely as a result of no detectable COX-2 migrating exactly with our positive control (27), and showed no influence of exercise. Reexamination of those blots (65) showed that the lower migrating band was also unchanged.

Statistical Analysis

Subject characteristics between the placebo and the COX-2 inhibitor groups were compared using a *t*-test. FSR, COX mRNA expression, and COX protein levels at rest and postexercise were compared with a two-way analysis of variance (group × time), with repeated measurements on time. A one-way analysis of covariance, considering resting FSR, was used to compare the postexercise FSR between the placebo and the COX-2 inhibitor groups. Post hoc comparisons were made with Tukey's test.

The fold change in COX mRNA between each group was compared using a *t*-test. Significance was accepted at $P < 0.05$. All data are presented as means ± SE.

RESULTS

There were no differences ($P > 0.05$) between the placebo and COX-2 inhibitor groups for age, height, weight, or one-repitition maximum (Table 1). The plasma [$^2H_5$]phenylalanine enrichments (tracer-to-tracee ratio, $m + 5/m + 0$) in the placebo group were similar during the preexercise (2.5 h: 0.0645 ± 0.0026; 3.5 h: 0.0657 ± 0.0032; 4.5 h: 0.0636 ± 0.0024) and postexercise (2.5 h: 0.0668 ± 0.0028; 3.5 h: 0.0673 ± 0.0029; 4.5 h: 0.0666 ± 0.0032) infusions. Similar plasma enrichments were also seen in the preexercise (2.5 h: 0.0691 ± 0.0041; 3.5 h: 0.0688 ± 0.0044; 4.5 h: 0.0691 ± 0.0039) and postexercise (2.5 h: 0.0724 ± 0.0025; 3.5 h: 0.0723 ± 0.0030; 4.5 h: 0.0710 ± 0.0025) infusions for the COX-2 inhibitor group.

The muscle tissue fluid and mixed muscle protein hydrolysate [$^2H_5$]phenylalanine enrichments are presented in Table 2. The plasma and muscle tissue fluid [$^2H_5$]phenylalanine enrichments reflect a steady-state delivery of tracer and equilibrated precursor pool during the mixed muscle protein synthesis period (i.e., 2–5 h of the infusion period) in both the placebo and COX-2 inhibitor groups. Mixed muscle protein FSR in response to exercise was not suppressed in the COX-2 inhibitor group compared with the placebo group ($P < 0.05$, main effect for exercise) (Fig. 2). In addition, there was no significant difference ($P > 0.05$) between the placebo (0.091 ± 0.005%/h) and COX-2 inhibitor (0.108 ± 0.014%/h) groups postexercise when controlling for resting FSR.

COX-1v1 and COX-1v2 mRNA levels in response to exercise were not different ($P > 0.05$) between the COX-2 inhibitor and placebo groups, whereas COX-2 mRNA was increased ($P < 0.05$) in the COX-2 inhibitor group (3.0 ± 0.9-fold) compared with the placebo group (1.3 ± 0.3-fold) (Fig. 3). However, COX-1v1 and COX-1v2 mRNA expression was significantly increased in both groups after exercise ($P < 0.05$, main effect for exercise) (Table 3). The COX-1b variants showed low-level expression in only a few of the subjects from each group before and after exercise (data not shown), which is similar to our previous findings (65). COX-1 and COX-2 protein levels were not influenced ($P > 0.05$) by the COX-2 inhibitor or the exercise bout (Fig. 4).

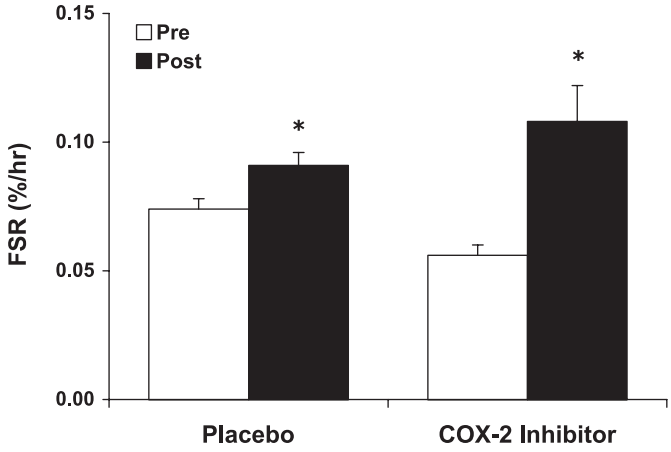

Fig. 2. Mixed muscle protein fractional synthesis rate (FSR) in the placebo and COX-2 inhibitor groups at rest and 24 h following the resistance exercise bout. *$P < 0.05$, main effect for exercise. Analysis of covariance revealed no significant difference ($P > 0.05$) between the placebo and COX-2 inhibitor groups postexercise when controlling for resting FSR.

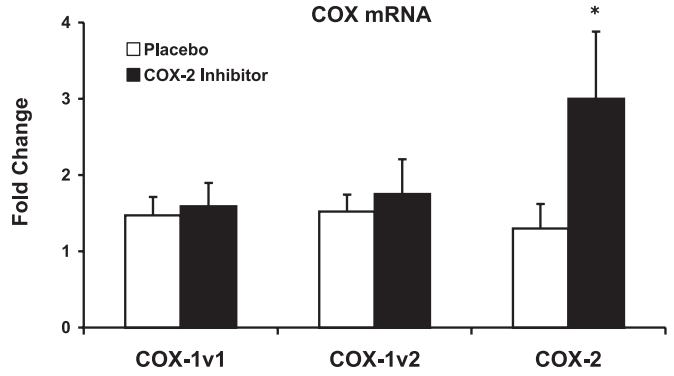

Fig. 3. Fold changes in mRNA levels of COX variant 1 (COX-1v1) and variant 2 (COX-1v2) and COX-2 in the placebo and COX-2 inhibitor groups from rest to 24 h following the resistance exercise bout. mRNA levels were normalized to GAPDH using the $2^{-\Delta\Delta C_T}$ relative quantification method. *$P < 0.05$ from placebo.

Table 3. *COX mRNA expression levels pre- and postexercise*

	Placebo			COX-2 Inhibitor		
	Pre	Post	%Δ	Pre	Post	%Δ
COX-1v1	3.60 ± 0.53	4.95 ± 0.81†	44	3.37 ± 0.64	4.84 ± 0.93†	46
COX-1v2	7.20 ± 0.83	10.36 ± 1.53†	48	6.92 ± 1.30	10.75 ± 2.80†	58
COX-2	0.21 ± 0.03	0.25 ± 0.06	32	0.18 ± 0.04	0.45 ± 0.13*	176

Values are means ± SE. COX-1v1 and COX-1v2, COX-1 variants 1 and 2, respectively. Numbers reflect the mRNA expression of the specific COX variant or isoform normalized to GAPDH in arbitrary units ($2^{-\Delta C_T} \times 10^5$) at rest (Pre) and 24 h following resistance exercise (Post). *$P = 0.06$ compared with Pre; †$P < 0.05$ main effect for exercise for both COX-1 variants.

DISCUSSION

The goal of the present study was to expand on our previous findings (58, 59, 61, 65) and further elucidate which COX enzyme(s) are involved in the regulation of skeletal muscle protein synthesis after exercise. Contrary to our hypothesis, the current findings show that the COX-2 isoform does not appear to be involved in the COX-mediated increase in muscle protein synthesis following resistance exercise in humans.

Our hypothesis for the current study was based in part on what has been shown in animal and cell culture investigations. Studies using animal models of muscle hypertrophy (3, 33), regenerative healing following injury (2, 16, 49–51), and cell culture experiments of myogenesis (30, 35) elegantly show the involvement of COX-2 in regulating these skeletal muscle processes. The lack of an inhibitory effect on postexercise muscle protein synthesis by the COX-2 inhibitor in the current study prompted us to reexamine the models employed in these animal studies. Synergist ablation over 14 days results in muscle hypertrophy of 80% (40%/wk), which is blunted by 75% with COX-2-selective inhibition (33). Comparatively, this amount of hypertrophy far exceeds the greatest rate in humans reported in the literature (~1%/wk) (56) and represents levels never seen with resistance training in humans of any duration. Also, edema appears to contribute 20–30% of the muscle mass gains in the synergist ablation model (33, 53), which does not happen in ambulatory or unloaded humans undergoing resistance training (5, 20, 26). Furthermore, 2 wk of regrowth from hindlimb suspension-induced atrophy is blunted in slow and fast muscles by 30–40% with a COX-2 inhibitor, when the rate of growth is 25%/wk (3). Considering the large discrepancy in the muscle growth response between these animal models and humans undergoing chronic resistance training, it is reasonable to expect that different mechanisms could regulate the hypertrophic process in the different models. The muscle-loading patterns across the studies also support this notion. The animal models of hypertrophy likely provide almost constant loading. Muscle loading in the current study was only about 3 min, and typical resistance training protocols provide this amount of stimulus to a muscle group three times/wk, albeit at a somewhat lower intensity.

Given the large discrepancies between the animal hypertrophy models and typical human resistance training protocols and responses, it is also not surprising that animal models of recovery from muscle injury induced by local freeze injury (2), laceration injury (49, 51), snake venom-induced injury (50), or ischemia-reperfusion injury (16) may not reflect the processes that regulate muscle protein synthesis and muscle hypertrophy after resistance exercise in humans. Overall, the COX-2 isoform in skeletal muscle appears to be more responsive to injury-related stimuli, which is consistent with what is known about COX-2 in most other tissues. This concept is generally supported by the large induction of skeletal muscle COX-2 protein levels in humans with septic myopathy (42).

The increase in COX-2 message in the COX-2 inhibitor group suggests the possibility that there was some COX-2 protein in the muscle during the 24 h following the resistance exercise that was blocked by the COX-2 inhibitor and resulted in a transcriptional response within the muscle. However, we did not detect any change in the level of COX-2 protein in the muscle 24 h postexercise, suggesting that COX-2 protein is present at basal levels or was transiently induced by the exercise bout. Whether or not enzymatically active COX-2 protein is present in human skeletal

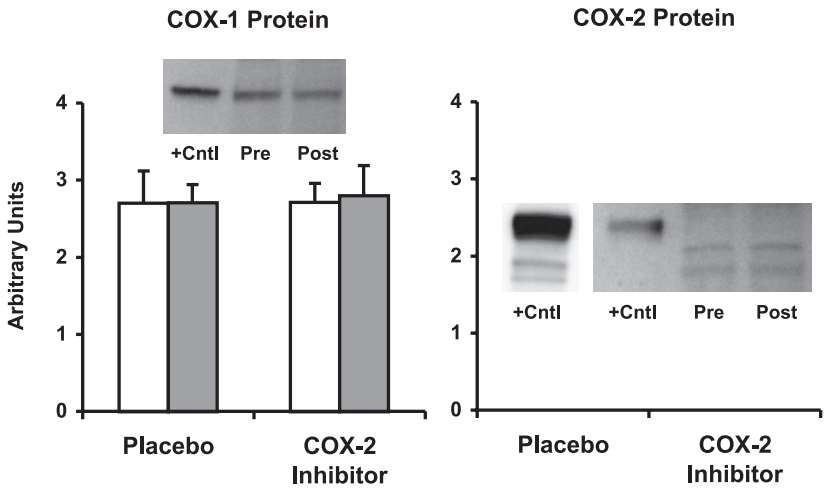

Fig. 4. COX-1 and COX-2 protein levels in the placebo and COX-2 inhibitor groups at rest (open bars) and 24 h following the resistance exercise bout (shaded bars). COX-2 protein levels were undetectable at rest or 24 h postexercise in either group (see MATERIALS AND METHODS for further description). Inserts in COX-1 and COX-2 graphs are representative Western blots with positive controls. The additional insert at *left* in the COX-2 graph is the human recombinant positive control run separately and loaded per the manufacturer's directions, with the lower 2 bands likely representing at least 2 versions of the glycosylated COX-2 protein (39). The amount of human recombinant positive control loaded in the representative COX-2 image at *right* is 0.3% of the manufacturer's directions.

muscle at basal levels is unclear (see MATERIALS AND METHODS and Fig. 4) (2, 16, 42, 54, 57, 65). Certainly, COX-2 mRNA levels are very low in resting human skeletal muscle (Table 3), since our previous study was unable to reliably detect COX-2 mRNA ($C_T >$ 34) (65) when less total RNA was used for the PCR analysis.

It appears that the COX pathways regulate a wide array of growth- and injury-related processes in skeletal muscle, and the specific isoform involved may vary depending on the stimulus applied to the muscle. The present results, coupled with our previous findings (61, 65), suggest that the COX-1 isoform may be responsible for the COX-mediated postexercise increase in muscle protein synthesis in humans. This explanation fits with the notion of COX-1 being a constitutively expressed protein that regulates normal housekeeping functions of cells, as protein synthesis would be considered in skeletal muscle cells. It should be noted the COX-2 inhibitor did not influence the COX-1 protein or mRNA levels, further supporting the selectivity of the drug. Unfortunately, to our knowledge, there are no COX-1-selective inhibitors for use in humans to determine whether COX-1 is involved exclusively in the COX-mediated muscle protein metabolism response following exercise.

Although the protein levels of COX-1 were not changed at 24 h after exercise in the 16 men studied, the COX-1 mRNA (variant 1 and 2) did significantly increase (45 and 53%, respectively). These findings coincide with our previous data from preexercise to 24 h after resistance exercise (65) when only the men ($n = 8$) in that study are considered (35 and 66%, respectively). The influence of chronic resistance training on COX-1 (and COX-2) protein levels needs to be examined and may provide insight into the role of the two isoforms on the hypertrophic response in humans. Additionally, given the relative high abundance of COX-1 variant 2 mRNA in human skeletal muscle and the responsiveness of this variant to exercise, further examination of this variant and the possible protein product is needed.

In conclusion, consumption of over-the-counter nonselective COX inhibitors blocks the increase in muscle protein synthesis after resistance exercise (61), and consumption of a prescription COX-2-selective inhibitor does not result in this same response. Thus, it is possible that the COX-mediated postexercise increase in muscle protein synthesis involves mainly the COX-1 isoform. Given the widespread use of nonselective COX inhibitors and the insight into the mechanisms that regulate skeletal muscle metabolism and hypertrophy that these drugs provide, additional studies focused on the COX pathways in skeletal muscle are clearly warranted.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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Excess exposure to insulin may be the primary cause of insulin resistance

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Excess exposure to insulin may be the primary cause of insulin resistance

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TO THE EDITOR: insulin resistance is either a precursor or a key component of numerous major diseases closely linked to the modern lifestyle, which is featured by overeating and/or lack of physical activities. These diseases include obesity, metabolic syndrome, type 2 diabetes mellitus, cardiovascular disorders, and many others. The impact of these problems on the global health and economy could not be more obvious now. The primary cause of insulin resistance is the positive energy imbalance due to overeating and/or lack of physical activities. However, the primary player that converts the positive energy imbalance into insulin resistance and the consequent hyperinsulinemia is still hotly debated. Establishment of this player may provide clear and specific targets for the prevention and treatment of insulin resistance and its associated health problems.

We have observed recently that the basal level of the classical Akt-dependent insulin signaling is increased in mice with insulin resistance and hyperinsulinemia induced by the high-fat diet (HFD) (5). Blunting the increased insulin signaling with the phosphatidylinositol 3-kinase inhibitor LY-294002 during the time when mice slept and did not highly demand insulin completely prevented the development of insulin resistance induced by the HFD (5). Both ectopic fat accumulation and oxidative stress in the liver and skeletal muscles were induced by the HFD, but the induction was prevented by the blockade of insulin signaling with LY-294002 (5). These results indicate that the increased basal insulin signaling causes insulin resistance and hyperinsulinemia through the promotion of ectopic fat accumulation and oxidative stress while working hard to maintain normal blood glucose levels. These results help explain why a majority of subjects with insulin resistance/hyperinsulinemia can maintain their blood glucose at normal or nearly normal levels without ever developing overt diabetes.

Furthermore, we have observed that hyperglycemia does not cause obvious insulin resistance, but administration of long-acting insulin (detemir) causes severe insulin resistance in mice (6). Administration of detemir mimics the continuous hyperinsulinemia induced by the HFD and is accompanied by the ectopic fat accumulation and oxidative stress in the liver and skeletal muscles (6).

In investigating the associated mechanisms, we have found that the mitochondrial production program is reduced by the excess exposure to insulin induced by either the HFD or administration of detemir (5, 6). Blockade of the increased basal insulin signaling reverses the suppression of mitochondrial production (5). Interestingly, macroautophagy (autophagy) may be also inhibited in mice with insulin resistance/hyperinsulinemia induced by either the HFD or administration of detemir (7). Autophagy is normally required for removing the aged/damaged macromolecules and cellular organelles, including mitochondria (7). In cultured hepatocytes, we have observed that prolonged exposure to insulin inhibits both mitochondrial biogenesis and mitophagy (autophagy of mitochondria) (7, 8). These observations imply that the suppressed production of new mitochondria via biogenesis and the reduced removal of aged/dysfunctional mitochondria via autophagy may be major contributors to the increased oxidative stress and insulin resistance induced by the HFD or administration of excess long-acting insulin.

In summary, our recent observations strongly support the notion that insulin may be the primary/necessary player that converts the positive energy imbalance into insulin resistance and associated diseases. Our observations echo some early findings by others that atherosclerosis does not occur or even reverses in the absence of insulin (1–4, 9). Thus, blockade of excess exposure to insulin may be a viable approach to prevent and/or reverse insulin resistance and its numerous associated diseases.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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