Effects of β-Hydroxy-β-methylbutyrate Free Acid and Cold Water Immersion on Expression of CR3 and MIP-1β following Resistance Exercise


Institute of Exercise Physiology and Wellness, Sport and Exercise Science, University of Central Florida, Orlando, FL 32816


Running Head: Effects of recovery treatments on CR3 and MIP-1β

Corresponding Author:
Maren S. Fragala, Ph.D.
Sport and Exercise Science
University of Central Florida
College of Education & Human Performance
P.O. Box 161250
Orlando, FL 32816-1250
407-823-1272
Maren.Fragala@ucf.edu

Copyright © 2014 by the American Physiological Society.
The inflammatory response to muscle damaging exercise requires monocyte mobilization and adhesion. Complement receptor type 3 (CR3) and macrophage inflammatory protein (MIP)-1β enables monocyte recruitment, adhesion, and subsequent infiltration into damaged muscle tissue. The purpose of this study was to examine the effects of cold water immersion (CWI) and/or β-hydroxy-β-methylbutyrate free acid (HMB-FA) on CR3 expression and MIP-1β concentration following four sets of up to ten repetitions of squat, dead lift, and split squat exercises at 70-80% 1-repetition maximum. Thirty-nine resistance-trained men (22.2 ± 2.5 y) were randomly divided into four groups: 1)Placebo (PL); 2)HMB-FA; 3)HMB-FA-CWI; 4)PL-CWI. The HMB-FA groups ingested 3 g·d⁻¹ and CWI groups submersed into 10-12°C water for 10 minutes following exercise. Blood was sampled at baseline (PRE), immediately post (IP), 30 minutes post (30P), 24 hours post (24P), and 48 hours post (48P) exercise. Circulating MIP-1β was assayed and CR3 expression on CD14+ monocytes was measured by flow cytometry. Without treatment, CR3 expression significantly elevated at 30P when compared to other time points (p=0.030-0.047). HMB-FA significantly elevated the percent of monocytes expressing CR3 between IP and 24P (p=0.046) and between IP and 48P (p=0.046). No time effect was observed for MIP-1β concentration. The recovery modalities showed to attenuate the rise in CR3 following exercise. Additionally, supplementation with HMB-FA significantly elevated the percent of monocytes expressing CR3 during recovery. Although the time course which inflammatory responses are most beneficial remains to be determined, recovery modalities may alter immune cell mobilization and adhesion mechanisms during tissue recovery.

Keywords: muscle damage, recovery, CD11b, Macrophage-1 antigen (Mac-1), supplement
INTRODUCTION

The mechanical stress of resistance exercise causes significant skeletal muscle damage which initiates a subsequent local inflammatory response (24, 25). This response results in a mobilization and infiltration of leukocytes to the damaged tissue (5, 33). Macrophage inflammatory protein (MIP)-1β and complement receptor type 3 (CR3) are part of the acute immune response that enables monocyte recruitment, adhesion, and subsequent infiltration into the damaged muscle tissue. CR3 is a β2 integrin that mediates leukocyte adhesion, migration, and phagocytosis in response to inflammatory stimuli (3, 29). CR3 contains a combination of pro-inflammatory cell surface receptors (CD11b and CD18) which facilitate the binding of monocytes to cell adhesion molecules on endothelial cells, allowing monocyte infiltration of the damaged muscle tissue (11, 40). Macrophage inflammatory protein (MIP)-1β is a critical pro-inflammatory chemokine for inducing inflammation and regulating tissue homeostasis (17, 31). MIP-1β exerts its effects by binding to cell surface receptors (CCR5) initiating a cascade of intracellular events that lead to numerous cell functions including chemotaxis, degranulation, phagocytosis, and mediator synthesis (17).

β-Hydroxy-β-methylbutyrate (HMB), a metabolite of the amino acid leucine, has shown to improve muscular recovery and reduce indices of muscle damage when supplemented in conjunction with resistance exercise (22, 45-47). Recently, HMB in free acid form (HMB-FA) has been shown to attenuate circulating tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine responsible for signaling the migration of neutrophils and macrophages to the site of muscle damage, following muscle damaging resistance exercise (42). Additionally, HMB appears to stimulate muscle protein synthesis through an up-regulation of the mammalian target of rapamycin (mTOR) (4, 20) and attenuate muscle protein degradation by inhibiting the
ubiquitin-proteasome pathway (37, 38). However, the mechanisms underlying the inflammatory contributions to tissue remodeling \textit{in vivo} are yet to be determined. Studies investigating the role of HMB on monocyte recruitment via CR3 and MIP-1β may better elucidate its role in altering the inflammatory cascade to muscle damage.

Cold water immersion (CWI) is a common modality used to enhance recovery from high intensity exercise. A recent meta-analysis investigating the efficacy of CWI showed benefits in alleviating symptoms of delayed-onset of muscle soreness (DOMS), reducing post-exercise elevations in creatine kinase (CK), and improving muscle power recovery (15). Some evidence also suggests that CWI may suppress cellular components of the immune response such as decreasing lymphocyte proliferation and down-regulating the immune cascade (35). Additionally, CWI has been shown to alter inflammation by blunting the rise in total number of leukocytes and CK following exhaustive intermittent exercise (30). However, to date, the effects of CWI on immune recruitment and adhesion are not fully understood.

The effects of CWI and HMB-FA on the modulation of CR3 and the pro-inflammatory chemokine, MIP-1β, have not previously been investigated. However, it conceivable that CWI and HMB-FA may alter immune cell mobilization and when combined may be incorporated into a feasible strategy to enhance recovery. Thus, the purpose of the present study was to investigate the effects of acute HMB-FA supplementation alone and in conjunction with CWI on circulating MIP-1β and monocyte expression of CR3 following muscle damaging resistance exercise. Specifically, we sought to examine the effect of CWI and HMB-FA on CD14+ monocyte CR3 expression and circulating MIP-1β concentration following an acute bout of high intensity resistance exercise. Examination of these markers may provide further insight into the immune
MATERIALS AND METHODS

Participants

Thirty-nine resistance-trained men (22.2 ± 2.5 y; 82.8 ± 10.0 kg; 1.7 ± 0.1 m; 11.9 ± 4.5 body fat %) with an average squat of 1.8 ± 0.3 times their body weight, and 6.0 ± 3.0 years of resistance training experience volunteered to participate in this randomized, double-blind, placebo-controlled study. None of the participants were competitive athletes, and all were recreationally lifting at study enrollment. Strict recruitment criteria were implemented to increase homogeneity of the sample, including between the ages of 18 and 35 years, a minimum of one year of resistance training experience, particularly with the squat, and the ability to squat a weight equivalent to their body weight. Following an explanation of all procedures, risks, and benefits, each participant gave his informed consent prior to participation in this study. The Institutional Review Board of the University approved the research protocol. Participants were not permitted to use any additional nutritional supplements or medications while enrolled in the study. Screening for nutritional supplements and performance enhancing drug use was accomplished via a health history questionnaire completed during participant recruitment. Participants were instructed not to partake in any additional recovery strategies while enrolled in the study including saunas, stretching routines, foam rollers, massages, additional hot/cold water therapy, etc.

Participants were randomly assigned to one of four treatment groups as enrolled in the study: β-hydroxy-β-methylbutyrate free acid only (HMB-FA), placebo only (PL), HMB-FA and cold water immersion (HMB-FA-CWI), and PL and CWI (PL-CWI). Experimental group
characteristics are displayed in Table 1. Groups did not differ in age, body mass, height, body
fat percentage, resistance training experience, or 1-RM strength.

***INSERT TABLE 1 HERE***

**Study Protocol**

Participants reported to the Human Performance Laboratory (HPL) on four separate
occasions (T1 – T4). During the first visit (T1), participants were tested for maximal strength (1-
RM) on the barbell back squat, dead lift, and barbell split squat exercises. Prior to the second
visit (T2), participants were instructed to refrain from all forms of exercise for a minimum of 72
hours. Participants were also instructed to report to the HPL during T2 – T4 in a 10-hour fasted
state at the same time each morning between 7:00 and 10:00 AM hours to reduce the potential
influence of diurnal variations. Participants were allowed to drink water prior to and during the
experimental testing. During T2, participants performed a lower body resistance exercise session
which consisted of four sets of the squat, dead lift, and barbell split squat exercises. The rest
interval between each set and between all exercises was 90 s. The squat exercise was performed
at 80% of the participant’s previously measured 1-repetition maximum (RM), while the dead lift
and barbell split squat exercises were performed at 70% of the participant’s previously measured
1-RM. Participants were encouraged to perform as many repetitions as possible, but not to
exceed 10 repetitions in any set. This protocol was utilized to simulate a typical high intensity
lower body training routine during a hypertrophy phase of training. Participants then reported
back to the HPL 24 (T3) and 48 hours (T4) post-exercise. During T3 and T4, participants only
performed four sets of the squat exercise using the same loading pattern and rest interval length
as T2. Participants were administered the supplement (or placebo) 30 minutes prior to exercise
on T2 – T4. On T2 and T3, participants also were administered the supplement (or placebo) at 2- and 6- hours following exercise. Therefore, participants in the HMB-FA groups received a total of 3 grams HMB-FA on T2 and T3, while receiving 1 gram HMB-FA on T4 (prior to resistance training only). Participants were also asked to maintain a dietary log during the study. Blood samples and subjective measures of soreness, pain, and recovery were obtained at five time points: baseline (PRE), immediately post-exercise (IP), and 30 minutes post exercise (30P) during T2, and 24- (prior to exercise on T3), and 48 h (prior to exercise on T4) post T2 (24P and 48P, respectively). The study protocol is depicted in Figure 1.

***INSERT FIGURE 1 HERE***

**HMB-FA Supplementation**

Each serving of HMB-FA and PL was provided in identical packets containing similarly flavored gel. The HMB-FA supplement consisted of 1 gram of β-hydroxy-β-methylbutyrate free acid, reverse osmosis water, debittering agent, orange flavor, stevia extract, and potassium carbonate. Each serving of PL consisted of an equivalent amount of litesse polydextrose, citric acid, corn syrup, 10% stevia powder, debittering agent, and orange flavoring. The HMB-FA and PL was obtained from Metabolic Technologies Inc. (Ames, IA). All HMB-FA and PL ingestion took place in the HPL and was witnessed by a study investigator to ensure 100% compliance. In addition, blood plasma HMB concentrations were analyzed by gas chromatography-mass spectrometry and performed by Metabolic Technologies Inc. using methods previously described to assess compliance and validate HMB in supplement packets (19).
Cold Water Immersion (CWI)

Following exercise, participants in the HMB-FA-CWI and PL-CWI groups were required to fully immerse their lower body into a metal tub (58.4 cm x 121.9 cm) filled 30 cm high with ice water at 10-12° C. Participants immersed in the water up to their umbilicus for 10-minutes. Reductions in femoral artery blood flow and muscle temperature have previously been reported following 10-minutes of CWI in both 8° C and 22° C water (7). Participants who were not in the CWI groups were required to remain in the HPL for the 10-minutes following exercise to ensure a similar post-exercise nutritional intervention opportunity among participants.

Performance Measures

Prior to each exercise session, participants performed a standardized warm-up consisting of 5 minutes on a cycle ergometer, 10 body weight squats, 10 body weight walking lunges, 10 dynamic walking hamstring stretches, and 10 dynamic walking quadriceps stretches. The 1-RM tests were performed using methods previously described by Hoffman (2006) (8). Each participant performed two warm-up sets using a resistance that was approximately 40-60% and 60-80% of his perceived maximum, respectively. Following this, 3-4 subsequent trials were performed to determine the 1-RM. A 3-5 minute rest period was provided between each trial. A certified strength and conditioning specialist observed each repetition to ensure proper range of motion, and verbal signals were given when the subject reached the appropriate final descent position. Trials not meeting the range of motion criteria for each exercise were discarded. The squat exercise required the participant to place an Olympic bar across the trapezius muscle at a self-selected location. Each participant descended to the parallel position which was attained when the greater trochanter of the femur reached the same level as the knee. The participant then
ascended until full knee extension. The dead lift exercise required the participant to grasp an Olympic bar slightly wider than shoulder width with the arms in a fully extended position. A closed, open, or alternating hand grip was allowed and kept consistent for each participant. From a flexed position, the participant extended his hips and knees until the body assumed an erect standing position. To minimize the risk for injury, the barbell split squat 1-RM was assessed only with the dominant leg forward using a prediction formula based on the number of repetitions performed to fatigue using a given weight (2). The barbell split squat required the participant to place an Olympic bar across the trapezius muscle at a self-selected location. The participant assumed an alternating leg stance with the dominant leg forward. For each repetition, the participant flexed the dominant knee until it was over the dominant foot. The trailing knee was lowered to the floor without making contact, while the torso remained erect. The participant pushed off with both legs to return back to the starting position.

**Blood Measurements**

During T2, PRE blood samples were obtained following a 15 minute equilibration period. Additional blood samples were also drawn IP and 30P. All blood samples were obtained using a 20-gauge Teflon cannula placed in a superficial forearm vein using a three-way stopcock with a male luer lock adapter. The cannula was maintained patent using an isotonic saline solution (Becton Dickinson, Franklin Lakes, NJ). IP blood samples were taken within 1 minute of exercise cessation. To standardize each participant following the resistance exercise protocol, participants remained in the supine position for the full 30 minutes recovery phase prior to the 30P blood sample being drawn, except for the participants in the CWI groups, who spent the first 10 minutes of the 30 minutes in the ice bath. All T2 blood samples were drawn with a plastic syringe while the participant was in a supine position. During T3 and T4, only pre-exercise
blood samples were drawn (24P and 48P, respectively) following a 15 minute equilibration period. These blood samples were obtained from an antecubital arm vein using a 20-gauge disposable needle equipped with a Vacutainer® tube holder (Becton Dickinson, Franklin Lakes, NJ). Each participant’s blood samples were obtained at the same time of day during each session.

All blood samples were collected into two Vacutainer® tubes, one containing no anti-clotting agent and the second containing K$_2$EDTA. The blood in the first tube was allowed to clot at room temperature for 30 minutes and subsequently centrifuged at 3,000×g for 15 minutes along with the remaining whole blood from the second tube. The resulting plasma and serum was placed into separate 1.8-mL microcentrifuge tubes and frozen at −80°C for later analysis.

**Cell Staining**

Blood samples were obtained from fresh, anti-coagulated (K$_2$EDTA), whole blood, and analyzed in duplicate. Erythrocytes were first lysed from 350 µl of whole blood with BD Pharm Lyse solution (BD Biosciences, Franklin Lakes, NJ) within 30 minutes of collection. Samples were then washed three times in staining buffer containing 1 x phosphate-buffered saline containing fetal bovine serum (BD Pharminigen; BD Biosciences) by centrifugation and aspiration. Leukocytes were then re-suspended in 100 µl BD Pharminigen. Direct staining methods were used to label CR3 and CD14. Allophycocyanin (APC) conjugated anti-CD11b (D12; BD Pharminigen), and PerCP Cy5.5 conjugated anti-CD14 (M5E2; BD Pharminigen) were used in the receptor labeling process. Surface staining was performed by adding 20 µl of directly conjugated APC-anti-CD11b and 5 µl of directly conjugated PerCP Cy5.5-anti-CD14 to the cell suspension and incubating in the dark for 30 minutes at 20°C. Cells were re-suspended in 1.0 ml of stain buffer for flow cytometry analysis.
Flow Cytometry

Flow cytometry analysis of stained cells was run on a BD C6 Accuri Flow Cytometer (BD Biosciences, San Jose, CA), equipped with BD Accuri analysis software (BD Biosciences). Forward and side scatter along with four fluorescent channels of data were collected using two lasers providing excitation at 488 and 640 nm. Monocytes were determined by initial gating based on forward and side scatter, followed by gating for CD14+ cells as also described by Tallone and colleagues (39). A minimum of 10,000 events, defined as CD14+ monocytes, were obtained with each sample (Figure 2).

Analysis of monocyte subpopulations was completed by quadrant analyses, in which CD14 was compared with CR3. Mean fluorescence of CR3 on CD14+ cells was recorded, representing the expression of CR3 per cell (6). Compensation for fluorescence spillover was set based on manufacturer recommendations (BD Biosciences).

Biochemical Analysis

Circulating levels macrophage inhibiting protein (MIP-1β) was assessed by Magpix (EMD Millipore, Billerica, MA, USA) via the human cytokine/chemokine panel one (EMD Millipore, Billerica, MA, USA). Samples were analyzed according to manufacturer’s guidelines with an average coefficient of variation 6.25%.

Creatine kinase (CK) was analyzed with the use of a spectrophotometer and a commercially available enzymatic kit (Sekisui Diagnostics, Charlottetown, PE, Canada) per manufacturer’s instructions. Determination of serum immunoreactivity values was determined
using a BioTek Eon spectrophotometer (BioTek, Winooski, VT, USA). To eliminate inter-assay variance, all samples for a particular assay were thawed once and analyzed in the same assay run by a single technician. All samples were run in duplicate with a mean intra-assay variance of 2.99%.

**Dietary Logs**

Participants were instructed to maintain their normal dietary intake leading up to the experimental trial. Participants were then instructed to record dietary intake as accurately as possible during T2 and T3. Dietary data was analyzed to identify differences between groups. Participants were instructed not to eat or drink (except water) within 10 hours of reporting to the HPL for subsequent visits. FoodWorks® Dietary Analysis software (McGraw Hill, New York, NY) was used to analyze the dietary recalls for total kilocalorie intake (kcal) and macronutrient distributions.

**Statistical Analysis**

The Shapiro-Wilk tests were used to verify the normal distribution of data, which were not normally distributed. The non-parametric Kruskal-Wallis one-way ANOVA test was performed to compare experimental groups at each time point. Subsequently, each experimental group was analyzed individually across time points using Friedman’s ANOVA as a non-parametric alternative to repeated measures ANOVA. Changes in dietary composition were analyzed using repeated measures ANOVA. Results were considered significant at an alpha level of $p \leq 0.05$. All data are reported as mean ± SD.
RESULTS

The lower-body resistance training protocol utilized in this study resulted in significant muscle damage as reflected by a significant decrease in performance and a significant rise in CK concentrations. However, no significant differences were noted between experimental groups for squat repetitions performed or CK concentrations between trials. All groups significantly decreased squat repetitions on T3 compared to T2 (−23.7 ± 22.2%). All groups significantly increased CK concentrations between PRE (136.8 ± 100.8 IU/L) and IP (205.5 ± 130.0 IU/L) and between IP and 24P (543.4 ± 331.2 IU/L). Analysis of dietary intake revealed no significant differences between groups for total kilocalorie intake, macronutrient distributions, or protein intake relative to body weight over the course of the study protocol. All groups consumed a daily average of 2675.9 ± 895.7 kcal, 299.9 ± 116.4 grams carbohydrate, 149.6 ± 75.0 grams protein, and 101.5 ± 48.1 grams fat. The average protein intake relative to body weight was 1.8 ± 0.9 grams/kg.

**CR3 Expression (reported as mean fluorescence) on CD14+ Monocytes**

Expression of CR3 on CD14+ monocytes is represented in Figure 3. No significant time point differences (p=0.192-0.952) were observed between groups. When experimental groups were analyzed individually using Friedman’s ANOVAs, PL significantly elevated expression of CR3 at 30P when compared to PRE (p=0.030), 24P (p=0.047), and 48P (p=0.047). No other significant within group time effects were noted.

***INSERT FIGURE 3 HERE***
Percent CR3+ Monocytes (CR3+/CD14+)

The percent of monocytes expressing CR3 is represented in Figure 4. No significant time point differences (p=0.406-0.755) were observed between groups. When experimental groups were analyzed individually using Friedman’s ANOVAs, HMB-FA significantly elevated the percent of monocytes expressing CR3 between IP and 24P (p=0.046) and between IP and 48P (p=0.046). No other significant within group time effects were noted.

***INSERT FIGURE 4 HERE***

MIP-1β Concentration

The concentration of MIP-1β is represented in Figure 5. No significant time point differences (p=0.299-0.823) were observed between groups. When experimental groups were analyzed individually using Friedman’s ANOVAs, no time effect was observed for any of the experimental groups.

***INSERT FIGURE 5 HERE***

DISCUSSION

The main findings of this investigation indicate that without treatment expression of CR3 on circulating monocytes increased at 30 minutes post-exercise. Treatments with HMB-FA and/or CWI showed no increases in CR3 expression following exercise or during recovery. However, the percent of monocytes expressing CR3 was significantly elevated in the HMB-FA group for up to 48 hours following resistance exercise. Additionally, no time or treatment effect was observed for any experimental group for MIP-1β concentrations.
Circulating MIP-1β did not appear to be strongly influenced by the resistance training protocol or the recovery treatments. Previous research has shown significant elevations in MIP-1β following a marathon run (21). However, similar to our results, previous studies have found no change in MIP-1β following 300 maximal eccentric contractions of the quadriceps (23) or 3 sets of 6-RM of the back squat, front squat, and leg extension exercises (32). As MIP-1β is a pro-inflammatory chemokine for regulating tissue repair (17, 31), we had expected to see elevations in circulating concentrations following the resistance training protocol that would be attenuated by recovery treatments. However, it is possible that the mode of exercise may dictate changes in circulating MIP-1β, where circulating changes are only observed with exhaustive aerobic type exercise. It is also possible that large observed inter-individual variations in circulating MIP-1β among participants may have masked any response to the exercise protocol or treatment effects. Large variations in circulating MIP-1β among participants is not uncommon (12) as MIP-1β is secreted by most immune cells and its production has shown to be induced by various other pro-inflammatory cytokines including TNF-α and IFNγ, and downregulated by IL-4 and IL-10 (17).

When no treatment was employed, the PL group significantly increased the expression of CR3 at 30 minutes post-exercise as compared to other time points. Previous investigations on the expression of CR3 (CD11b/CD18) and its component, CD11b, following exercise have yielded conflicting results. Pizza and colleagues (1996) showed CD11b expression was elevated above baseline levels at 24, 72, and 96 hours following eccentric exercise of the forearm flexors of untrained subjects, while Saxton and colleagues (2003) observed no changes in CD11b expression up to 72 hours following a bout of eccentric isokinetic contractions of the quadriceps or 40 minutes of high-intensity stepping exercise in recreationally active subjects. Additionally,
others have reported no changes in CD11b expression following sub-maximal cycle ergometry (14) and running (10). Conflicting findings are likely due to differences in training status of participants, as training status has been previously shown to impact the expression of CR3 on immune cells (10, 27). As CR3 mediates leukocyte adhesion, migration, and phagocytosis in response to inflammatory stimuli (3, 29), it is possible that the observed elevation at 30 minutes post-exercise without treatment in the current study would result in enhanced monocyte activity following exercise. Follow-up studies are needed to examine actual monocyte infiltration following high intensity resistance exercise in relation to the expression of CR3.

The HMB-FA, CWI, and combination of the two treatments all similarly attenuated the rise in CR3 expression observed in the PL group at 30 minutes post-exercise. Additionally, the HMB-FA group significantly elevated the percent of monocytes expressing CR3 for up to 48 hours following resistance exercise. To our knowledge, no studies have investigated the effects of HMB or CWI on CR3 and MIP-1β expression in vivo. However, in vitro studies in chicken and fish models have shown increased macrophage cell proliferation and phagocytosis suggesting the potential for HMB to be used as a possible dietary immunomodulator (26, 36). Additionally, leucine appears to exert a greater effect on immune function as compared to other branch chained amino acids (13), which has been partly explained by the differential ability of leucine to stimulate muscle protein synthesis through an up-regulation of mTOR (16, 18). HMB, a metabolite of leucine, may play a major role in the modulation of immune function. Recent in vivo research by Townsend and colleagues (2013) also showed that HMB-FA attenuated circulating TNF-α, another pro-inflammatory cytokine, immediately post-exercise and attenuated TNF-α receptor expression at 30 minutes post-exercise. It appears that HMB-FA may be effective in altering the immune response following resistance exercise. Contrary to our findings
for CR3 expression, Townsend et al. (2013) did not observe any differences in circulating TNF-α or its receptors with CWI treatment. An important consideration to the interpretation of our findings is the relatively high daily protein consumption (1.8 ± 0.9 grams/kg) of participants. While groups did not differ in daily protein consumption, prior research has shown that protein supplementation may positively impact post-exercise CK concentrations (9). Additionally, incremental exercise to exhaustion has shown to increase leukocyte concentration in conjunction with elevated levels of CD11b (44), while CWI has shown to blunt the rise in total number of leukocytes post-exercise (30). It may be that this attenuation in leukocyte concentration may also attenuate the rise in CR3 accompanied with muscle damaging exercise.

Limited research has been conducted on CR3 expression on monocytes in response to resistance training, but the present results may help us understand the in vivo leukocyte infiltration processes necessary for tissue repair. Although the treatment strategies employed in this study attenuated the rise in CR3 expression following exercise and HMB-FA showed to significantly elevate the percent of monocytes expressing CR3 for up to 48 hours following resistance exercise, the effects of the subsequent inflammatory events are yet to be determined. An increased expression of CR3 likely facilitates the infiltration of neutrophils and monocytes into the exercise-damaged muscle as a necessary stage in skeletal muscle repair, however neutrophils may also play a role in furthering muscle damage by releasing oxygen free radicals and proteases which potentially cause additional tissue injury (1, 41). Neutrophils are the first subpopulation of leukocytes to appear at the injury site (28), yet the time points in which pro-inflammatory and anti-inflammatory cytokines become most beneficial for recovery have yet to be determined (43). Thus, results should be interpreted as a component of the complex multifactorial series of events in the entire inflammatory process of tissue repair. Future research
is needed to better understand the timeline of CR3 and MIP-1β expression following resistance exercise relating to the magnitude of muscle repair.

**Perspectives and Significance**

This study is the first to investigate the effects of traditional recovery modalities on CR3 expression and MIP-1β concentrations following resistance exercise. The significant peak expression of CR3 at 30 minutes post-exercise was attenuated by HMB-FA, CWI, and the combination of the two treatments. Treatments with HMB-FA and/or CWI showed no increases in CR3 expression following exercise or during recovery. However, supplementation of HMB-FA significantly elevated the percent of monocytes expressing CR3 for up to 48 hours following resistance exercise. No time or treatment effect was observed for any experimental group for MIP-1β concentrations. Research on recovery modalities has focused on altering or alleviating the inflammatory process following acute exercise-induced muscle damage. However, it is not yet known at what time point during the acute inflammatory response it is most sensible to interfere with the natural repair progress, or if it is advisable to alter it at all. An understanding of the β2 integrin, CR3, and its response to resistance training provides a unique insight into the inflammatory response. The treatment modalities in this study showed to attenuate the rise in CR3 expression at 30 minutes post-exercise and HMB-FA showed to elevate the percent of monocytes expressing CR3 between IP and 24P and between IP and 48P; however this alleviation did not contribute to a more rapid recovery or improve subsequent performance.

**ACKNOWLEDGEMENTS**

This study was funded by a grant from Metabolic Technologies Inc.
REFERENCES


**FIGURE LEGEND**

**Figure 1.** Study Protocol. Supplement Time Points: 1, 4 & 7=30 min prior to exercise; 2 & 5=2 hr post exercise; 3 & 6=6 hr post exercise. Assessment Time Points: PRE=baseline, IP=immediately post exercise, 30P=30 min post exercise, 24P=24 hr post T2 exercise, 48P=48 hr post T2 exercise.

**Figure 2.** Gating procedures for CD14+ cells. (A.) CD14+ cells selected from the specified region based on forward (FSC) and side scatter (SSC) properties. (B.) Two dimensional histogram displaying fluorescence characteristics of cells in selected region. Cells positive for CR3 and CD14 are displayed in the upper right quadrant.

**Figure 3.** Acute effects of β-hydroxy-β-methylbutyrate free acid and/or cold water immersion on expression (mean fluorescence) of complement receptor 3 (CR3) on CD14+ monocytes. Treatment groups: PL=Placebo; HMB-FA=β-hydroxy-β-methylbutyrate free acid only; PL-CWI=Cold water immersion only; HMB-FA-CWI=β-hydroxy-β-methylbutyrate free acid and cold water immersion. Time points: PRE=Baseline; IP=Immediately post-exercise; 30P=30 min post-exercise; 24P=24 hours post-exercise; 48P=48 hours post-exercise. * PL significantly elevated expression of CR3 at 30P when compared to PRE (p=0.030), 24P (p=0.047), and 48P (p=0.047).

**Figure 4.** Acute effects of β-hydroxy-β-methylbutyrate free acid and/or cold water immersion on the percent of monocytes expressing CR3 (CR3+/CD14+). Treatment groups: PL=Placebo; HMB-FA=β-hydroxy-β-methylbutyrate free acid only; PL-CWI=Cold water immersion only; HMB-FA-CWI=β-hydroxy-β-methylbutyrate free acid and cold water immersion. Time points: PRE=Baseline; IP=Immediately post-exercise; 30P=30 min post-exercise; 24P=24 hours post-exercise; 48P=48 hours post-exercise. * HMB-FA significantly elevated the percent of monocytes expressing CR3 between IP and 24P (p=0.046). # HMB-FA significantly elevated the percent of monocytes expressing CR3 between IP and 48P (p=0.046).

**Figure 5.** Acute effects of β-hydroxy-β-methylbutyrate free acid and/or cold water immersion on MIP-1β. Treatment groups: PL=Placebo; HMB-FA=β-hydroxy-β-methylbutyrate free acid only; PL-CWI=Cold water immersion only; HMB-FA-CWI=β-hydroxy-β-methylbutyrate free acid and cold water immersion. Time points: PRE=Baseline; IP=Immediately post-exercise; 30P=30 min post-exercise; 24P=24 hours post-exercise; 48P=48 hours post-exercise.

**Table 1.** Experimental Group Characteristics. Treatment groups: PL=Placebo; HMB-FA=β-hydroxy-β-methylbutyrate free acid only; PL-CWI=Cold water immersion only; HMB-FA-CWI=β-hydroxy-β-methylbutyrate free acid and cold water immersion. RM=Repetition maximum. Data presented as mean ± SD.
<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>HMB-FA</th>
<th>PL-CWI</th>
<th>HMB-FA-CWI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n=10</td>
<td>n=9</td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>Age (y)</td>
<td>23.8 ± 3.0</td>
<td>21.2 ± 1.3</td>
<td>22.5 ± 3.0</td>
<td>21.2 ± 1.7</td>
</tr>
<tr>
<td>Body Mass (kg)</td>
<td>85.7 ± 5.4</td>
<td>80.4 ± 13.3</td>
<td>77.1 ± 7.7</td>
<td>87.6 ± 9.9</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.78 ± 0.06</td>
<td>1.77 ± 0.08</td>
<td>1.71 ± 0.07</td>
<td>1.71 ± 0.22</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>13.0 ± 3.4</td>
<td>12.3 ± 4.2</td>
<td>10.9 ± 4.1</td>
<td>11.6 ± 6.1</td>
</tr>
<tr>
<td>Resistance Training Experience (y)</td>
<td>7.6 ± 4.2</td>
<td>5.3 ± 1.3</td>
<td>5.7 ± 3.4</td>
<td>5.3 ± 2.0</td>
</tr>
<tr>
<td>1 RM Squat (kg)</td>
<td>148.0 ± 30.9</td>
<td>136.1 ± 36.4</td>
<td>148.7 ± 31.9</td>
<td>152.5 ± 17.4</td>
</tr>
<tr>
<td>1 RM Squat/Body Mass</td>
<td>1.72 ± 0.34</td>
<td>1.68 ± 0.29</td>
<td>1.94 ± 0.42</td>
<td>1.75 ± 0.20</td>
</tr>
<tr>
<td>1 RM Deadlift (kg)</td>
<td>151.4 ± 26.7</td>
<td>146.0 ± 39.0</td>
<td>157.3 ± 20.3</td>
<td>166.1 ± 20.0</td>
</tr>
<tr>
<td>1 RM Split Squat (kg)</td>
<td>101.4 ± 12.7</td>
<td>97.0 ± 16.8</td>
<td>108.2 ± 22.2</td>
<td>114.8 ± 16.9</td>
</tr>
</tbody>
</table>