

Effect of NSAID on Muscle Injury and Oxidative Stress

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Key words

- exercise
- drugs
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- protein oxidation

Abstract

Indirect markers of muscle damage and delayed onset muscle soreness were examined and correlated to changes in oxidative stress, plasma antioxidant potential, and use or nonuse of non-steroidal anti-inflammatory drugs in 60 ultra-marathoners following the Western States Endurance Run. Blood was collected prior to and immediately following the race and analyzed for muscle damage by creatine phosphokinase and oxidative stress by F₂-isoprostanes, protein carbonyls, and lipid hydroperoxides and antioxidant potential by the ferric reducing ability of plasma. Subjects recorded delayed onset muscle soreness during the week following the race. Lipid hydroperoxide

concentrations were unchanged, but F₂-isoprostanes, protein carbonyls, ferric reducing ability of plasma, creatine phosphokinase, and delayed onset muscle soreness increased significantly posttrace. Protein carbonyls were significantly higher posttrace in nonsteroidal anti-inflammatory drug users versus nonusers ($p < 0.05$). However, there was no difference between users and non-users for all other markers. Posttrace creatine phosphokinase concentrations were not correlated with oxidative stress markers but were correlated with changes in delayed onset muscle soreness. Based upon these findings, caution should be used when consuming nonsteroidal anti-inflammatory drugs during ultra distance events.

Introduction

An acute bout of long-duration running has been shown to increase oxidative stress [26,30]. Additionally, running has an eccentric component which can result in physical damage to the muscle ultra structure resulting in release of enzymes such as creatine phosphokinase (CPK) [10]. The potential link between oxidative stress and muscle damage occurs after infiltration of the damaged site by phagocytic cells. These cells release reactive oxygen species (ROS) which are thought to be involved in the progression of the initial injury by further damaging cell membranes and generating oxidative stress [34,37]. One of the major problems in the investigation of the link between ROS and muscle injury is the lack of a stable and reliable indicator of oxidative stress. Most often, indicators such as the reduced-oxidized glutathione ratio or malondialdehyde (MDA) have been used to link or examine ROS derived oxidative stress and muscle damage [16,34]. In particular, unless analyzed by HPLC, the MDA assay suffers from reliability problems

due to non-specificity [27]. Measurement of protein carbonyls (PC) have been found to be a stable marker of oxidative stress and to parallel oxidized proteins in working muscle [23]. Although different from the exercise used in the present study, Lee et al. [22] specifically found PC to be increased within 48 hours of initial eccentric muscle damage. Primarily, the measurement of F₂-isoprostanes are generally regarded as the best indirect index of oxidative stress and are a sensitive and stable marker of oxidative stress when measured by gas chromatography mass spectrometry. F₂-isoprostanes are prostaglandin-like compounds produced by non-cyclooxygenase free radical mediated lipid peroxidation of arachidonic acid [27]. Additionally, F₂-isoprostanes have been minimally examined in regard to muscle damage [18].

A second major problem in investigating the link between exercise-induced muscle damage and oxidative stress has been the mismatch in timing of peak plasma levels for markers of muscle damage (most common, CPK that peaks 1–3 days following exercise) and oxidative stress markers

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Bibliography

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which usually peak within minutes to a few hours following exercise [22,25]. Ultramarathon race events allow the relationship between muscle damage, perceptions of muscle soreness, NSAID use, and oxidative stress to be tested in an extreme exercise environment of long lasting duration. Elevations in oxidative stress markers and significant muscle damage should occur within the first few hours of the race and then be maintained for 20–30 hours when correlations with CPK could be tested at the end of the race. In support of this concept, Koller et al. [21] found that there is considerable muscle protein leakage in ultramarathoners and considerable slow-twitch skeletal muscle fiber damage after prolonged strenuous endurance exercise. This point in the production of muscle damage markers should exist when oxidative markers are still elevated [25]. It is unclear whether exercise-induced muscle damage causes changes in oxidative stress markers in the blood and what effect NSAID use has upon these markers.

Ultramarathoners may consume NSAIDs in an attempt to prevent muscle damage and soreness and thus enhance performance. However, NSAID use along with strenuous exercise may increase gut permeability and enhance endotoxin leakage from the gut into the blood resulting in inflammation and oxidative stress, but these relationships have not yet been verified using appropriate research designs [1,3,8]. In support of this, we recently reported that inflammatory plasma cytokines were two to three times greater in runners reporting use of NSAID following a 160-km race event compared to nonusers [31]. As stated previously, we have found bouts of long-duration running to increase oxidative stress [26,30]. To our knowledge, the effect of NSAID use upon oxidative stress during strenuous bouts of exercise has not been examined.

We hoped to improve upon previous studies which have attempted to link muscle damage and oxidative stress by using F₂-isoprostanes and PC as oxidative stress markers. Furthermore, we examined whether NSAID use during the race would influence any race outcome. There are some limitations to this study in that NSAID use was not controlled either prior to or during the race and plasma CPK was the sole biochemical indicator of muscle damage. Although routinely used to indicate muscle damage, CPK has been found to correlate poorly with actual changes in muscle histology [4]. Another limitation to this study is that no prerace dietary assessment or control was done. Despite this, we have not found any significant differences in macronutrient or antioxidant vitamin intake between groups of athletes in several prior studies we have conducted [26,30]. We hypothesized that plasma CPK, delayed onset of muscle soreness (DOMS), and oxidative stress markers would be positively correlated and that NSAID use would significantly diminish these markers by reducing inflammation.

Materials and Methods

Subjects and race description

Seventy-one experienced ultramarathoners were recruited and provided prerace blood samples. Of these, 60 completed the race and provided postrace blood samples. Subjects included both males (n = 45) and females (n = 15) without age restriction. Informed consent was obtained from each subject, and the experimental procedures were in accordance with the policy statements of the institutional review board of Appalachian State University. The 160-km Western States Endurance Run is a

Table 1 Flow diagram of outcome measure

Prerace	Ht, Wt, %BF, questionnaire, blood (CPK, F2s, LH, PC, FRAP), DOMS
↓	↓
Race day (90 km)	Wt
↓	↓
Race day (finish)	Wt, questionnaire, blood (CPK, F2s, LH, PC, FRAP)
↓	↓
1-d postrace	DOMS
↓	↓
2-d postrace	DOMS
↓	↓
3-d postrace	DOMS
↓	↓
4-d postrace	DOMS
↓	↓
5-d postrace	DOMS
↓	↓
6-d postrace	DOMS
↓	↓
7-d postrace	DOMS

point-to-point trail run in the Sierra Nevada Mountains of northern California, and is regarded as one of the most arduous organized running events in the United States. The race starts at Squaw Valley, California (1890 m altitude) and finishes at Auburn, CA, USA (366 m). The race starts at 5:00 a.m., and runners must reach the finish line within 30 hours to be eligible for an award.

Research design

Subjects provided blood the morning before the race. Upon arriving, subjects sat quietly for 15 min prior to having blood samples drawn from the antecubital vein with subjects in the seated position. Prerace body mass and percent body fat (3-site skinfolds) were measured, and subjects filled in a questionnaire on basic demographics, training history, and use of supplements during training. On race day, body mass was measured at the 90-km aid station and within 5–10 minutes postrace at Auburn. Postrace blood samples were provided immediately upon each subject finishing the race (within five min) from the antecubital vein with subjects in the seated position. Upon finishing, each subject was met by a member of the research team and escorted to the medical tent to ensure compliance. The within five minutes postrace blood sampling time was chosen because we have observed from previous studies that the oxidative stress markers rapidly decline after this time point [26,30]. Subjects completed a postrace questionnaire indicating self-selected use of medications and supplements during the race. Athletes were categorized as NSAID users if they reported use during the race, and nonusers if they reported complete avoidance of NSAID during the race. As discussed in the introduction and discussion sections as limitations, subjects did not adhere to any dietary or medicinal restrictions prior to or during the race. Following is a flow diagram of the study protocol (● Table 1).

DOMS and CPK

Subjects recorded muscle soreness prior to and for seven days immediately following the race using a 10-point Likert scale. Only postrace days one, three, five, and seven were reported because these were the only days when DOMS values correlated

Table 2 Subject characteristics (n=60, with 45 males and 15 females) (mean \pm SEM; range)

Variable	Mean \pm SEM	Range
Age (yr)	45.3 \pm 1.1	28–67
Height (m)	1.75 \pm 0.01	1.55–1.96
Prerace body mass (kg)	68.5 \pm 1.4	44.6–96.4
▶ 90 km (kg)	69.7 \pm 1.3	46.7–97.5
▶ 160 km (kg)	69.1 \pm 1.3	46.7–97.5
Body fat (%)	15.6 \pm 0.6	5.0–26.0
Running history (yr)	13.9 \pm 1.3	1–42
Ultramarathons raced (number)	34 \pm 69	1–378
Running distance (km/week)	80.5 \pm 3.4	22.4–144
Race time, 160 km (h)	26.3 \pm 0.4	19.1–29.9

significantly with CPK postrace. Runners were asked to supply a number that best described any general feeling of painful, sore, aching leg muscles using this scale: 1 (no soreness), 2.5 (dull, vague ache), 4 (slight soreness), 5.5 (more than slight soreness), 7 (sore), 8.5 (very sore), and 10 (unbearably sore). Plasma CPK was measured using an LX-20 clinical analyzer (Beckman, Brea, CA, USA). Plasma volume changes were estimated using the method of Dill and Costill [13].

Oxidative stress indicators

F₂-isoprostanes

Plasma F₂-isoprostanes were determined using gas chromatography mass spectrometry according to the methodology of Morrow [28]. Briefly, free F₂-isoprostanes were extracted from one mL of plasma. One to five pmol of deuterated [²H₄] PGF₂ internal standard was added and the mixture vortexed. This mixture was then added to a C₁₈ Sep Pak column, followed by silica solid phase extractions. F₂-isoprostanes were converted into pentafluorobenzyl esters, subjected to thin layer chromatography, and then converted to trimethylsilyl ether derivatives. Samples were then analyzed by a negative ion chemical ionization GC-MS using a Nermag R10-10C mass spectrometer (Delsi Nermag, Argenteuil, France) interfaced with an Agilent computer system.

Total plasma antioxidant potential

Total plasma antioxidant potential was determined by the ferric reducing ability of plasma (FRAP) assay according to the methodology of Benzie [5]. The basis of this assay is that water soluble reducing agents (antioxidants) in the plasma will reduce ferric ions to ferrous ions, which then react with an added chromogen. Samples and standards were analyzed in duplicate, and FRAP values were expressed as vitamin C equivalents as determined by linear regression from a vitamin C standard curve (0–1000 μ mol). Intra-assay and inter-assay coefficients of variation were less than 10% and 3%, respectively.

Lipid hydroperoxides

Lipid hydroperoxides (LH) of duplicate samples were determined after chloroform extraction using spectrophotometric analysis and a kit (#705002) obtained from Cayman Chemical (Ann Arbor, MI, USA). Samples and standards were analyzed in duplicate, and LH concentration was determined from a linear regression line generated from the standard curve of cumene hydroperoxide. Intra-assay and inter-assay coefficients of variation were less than 10% and 6%, respectively.

Protein carbonyls

PC were determined using the dinitrophenolhydrazine (DNPH) spectrophotometric method [22]. Briefly, plasma protein concentration was determined and adjusted to 4 mg/ml protein with phosphate buffer. Samples were run through columns containing Sephadex G-10 and rinsed with 2 N HCl. The effluent was collected and the absorbance determined at 360 nm. All samples were analyzed in duplicate and compared to the change in absorbance with and without DNPH. Values are expressed as molar quantities using the extinction coefficient of 22 000 m⁻¹·cm⁻¹. Intra-assay and inter-assay coefficients of variation were less than 10% and 7%, respectively.

Statistical analysis

Data are expressed as means \pm SEM. A 2-way ANOVA was originally used to test differences in all variables. This design did not detect any differences in treatments but only time effects. Therefore, a 1-way ANOVA design was used with Bonferroni post test comparisons ($p \leq 0.025$) to examine changes from prerace to postrace for all variables. The Bonferroni correction was used to tighten p values and thus avoid the chance of a type-1 error when making the multiple comparisons. Comparisons between genders, age differences, and use of supplements prior to and during the race were compared in NSAID users and nonusers for all variables using Student's *t*-tests. Pearson product-moment correlations were used to test the relationship between changes in all measured outcomes. All statistical analyses were done using Instat version 1.01 (GraphPAD Software, San Diego, CA, USA) and SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results



Sixty of 71 subjects completed the 160-km race event (race time, 27.0 \pm 0.4 h). Due to losses incurred during assays, total numbers are not always reflective of these 60 subjects. Subject characteristics in **Table 2** indicate the subjects were highly experienced ultramarathoners. Male and female runners did not differ significantly in race time or any of the other variables measured in this study except for body mass (72.7 \pm 1.2 versus 55.2 \pm 1.1 kg), height (1.78 \pm 0.01 versus 1.64 \pm 0.01 m), and percent body fat (14.3 \pm 0.7 versus 19.4 \pm 1.0%, respectively). Thus, male and female runners were combined for this data analysis. It is interesting that percent body fats are higher than what might be expected for someone able to train and run an event of this difficulty, but we have observed that ultra events tend to attract a segment of the older population. Plasma volume did not change appreciably ($-1.5 \pm 0.3\%$), and body mass was maintained near prerace levels (**Table 2**). Use of NSAIDs during the race was reported by 72% of the athletes. In the NSAID group, ibuprofen was used by all athletes except aspirin by two runners, naproxen by one, and cox-2 inhibitors in three runners.

CPK was significantly elevated postrace in both NSAID users (n=36) and nonusers (n=24) ($p < 0.001$ and $p < 0.05$), respectively. However, NSAID users and nonusers showed no differences in CPK (**Fig. 1**) or DOMS (**Fig. 2**). Interestingly, reported DOMS was significantly higher 1-d postrace in NSAID ($p < 0.05$) users (n=36) but not in nonusers (n=24) (**Fig. 2**). Also of interest is the high prerace DOMS rating. We suspect this could be from the continual training that ultramarathoners accomplish. F₂-isoprostanes were significantly increased 22% postrace ($p = 0.03$) for all subjects combined, but there were no group dif-

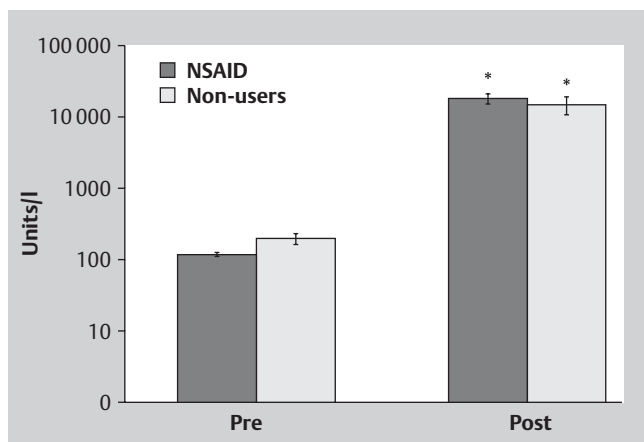


Fig. 1 CPK pre- and post-race. CPK was significantly increased in both NSAID ($n = 36$) and nonuser groups ($n = 24$) ($p < 0.001$ and $p < 0.05$), respectively. Group differences were not significant. * Significantly different from pre-exercise. Values are means \pm SEM.

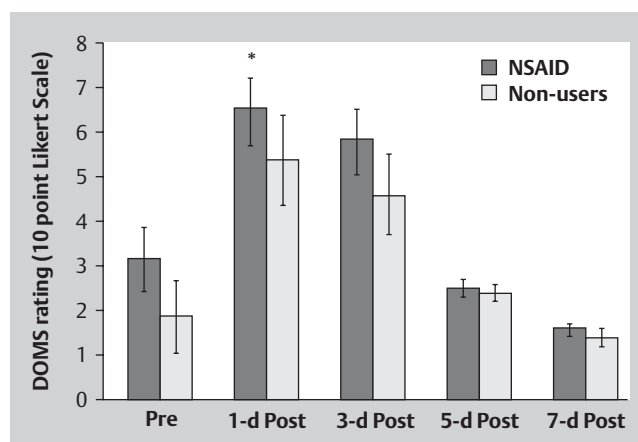


Fig. 2 DOMS pre- and post-race. DOMS was significantly higher in NSAID ($n = 36$) 1-d post-race but not at any time in non-user groups ($n = 24$) ($p < 0.05$). * Significantly different from pre-exercise. Values are means \pm SEM.

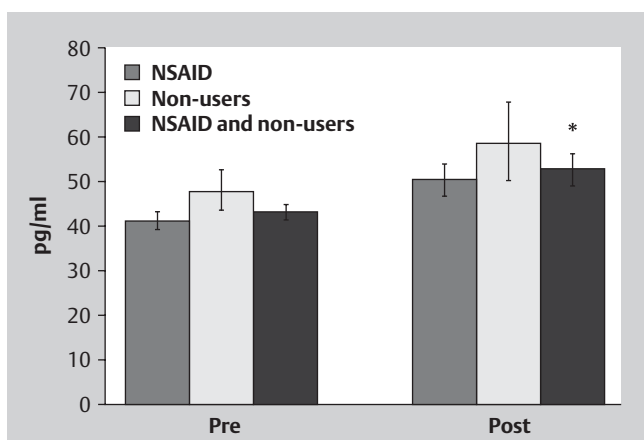


Fig. 3 F₂-isoprostanes over time in NSAID and nonuser groups and with both groups combined. F₂-isoprostanes were not significantly increased in either NSAID ($n = 36$) or nonuser ($n = 14$) groups. However, when both groups were combined ($n = 48$), there was a significant increase postexercise ($p = 0.03$). * Significantly different from pre-exercise. Values are means \pm SEM.

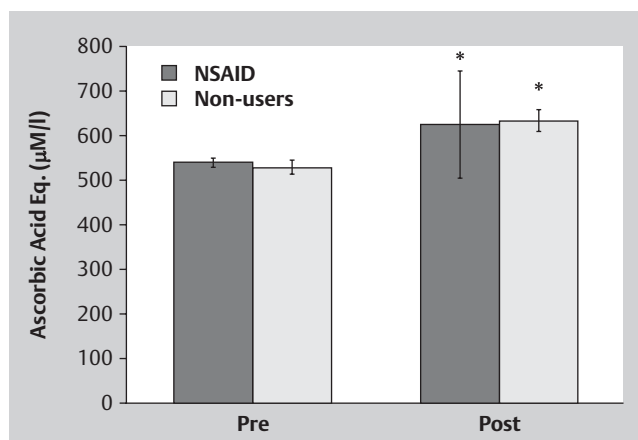


Fig. 4 Ferric reducing ability of plasma (FRAP) values over time in NSAID ($n = 36$) and nonuser ($n = 24$) groups. FRAP was significantly increased in both NSAID and nonuser groups ($p < 0.01$), but not between groups. * Significantly different from pre-exercise. Values are means \pm SEM.

ferences (NSAID, $n = 36$) and (nonuser, $n = 14$) (● Fig. 3). FRAP was significantly elevated ($p < 0.01$) in both NSAID users ($n = 36$) and nonusers ($n = 24$), but group differences were not detected (● Fig. 4). LH concentrations were not significantly altered in any group by exercise or NSAID use ($n = 36$) or nonuser ($n = 24$) (● Fig. 5). Interestingly, PC concentrations were increased postexercise ($p \leq 0.001$) versus pre-exercise in the NSAID group ($n = 43$) and were significantly different postexercise from the nonuser group ($n = 17$) (● Fig. 6).

CPK concentration was not correlated with any oxidative stress marker but was correlated with changes in DOMS (1-d post-race, $p = 0.048$, $r = 0.257$; 3-d post-race, $p = 0.035$, $r = 0.272$; 5-d post-race, $p = 0.027$, $r = 0.286$; and 7-d post-race, $p = 0.038$, $r = 0.269$).

Discussion

▼ In agreement with our hypothesis, perceptions of muscle soreness, muscle damage as assessed by CPK, oxidative stress, and plasma antioxidant potential were all significantly increased by

the race. Contrary to our hypothesis, NSAID use did not diminish any of these markers and actually increased PC concentrations. NSAID use also resulted in a significantly increased perception of DOMS 1-d post-race compared to nonuser. However, this may potentially be a spurious finding as we were unable to find any other studies to support this observation. We also examined whether there were any correlations between age, finishing time, and weekly training mileage with post-race oxidative stress measures. We did not find any relationship between any of these comparisons.

As noted in the introduction, we did not conduct a diet analysis prior to or during the race and this is certainly a limitation. However, we did ask whether subjects used any type of supplement routinely prior to the race or during the race. Although we did not inquire as to the type or amount of the supplements used, none of our race outcomes were different at any time between supplement users or nonusers (data not reported). Antioxidant supplements in particular have been known to influence oxidative stress parameters [24].

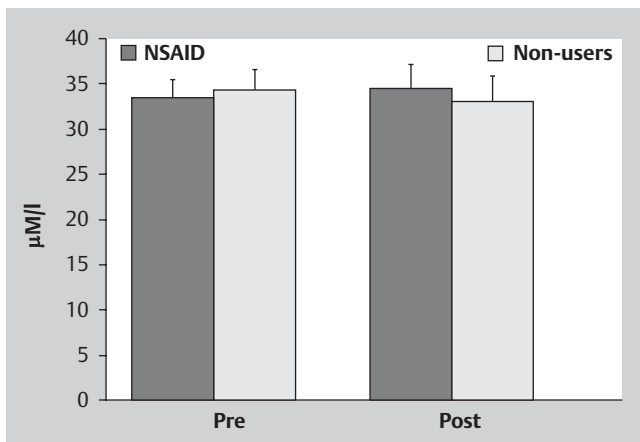


Fig. 5 Lipid hydroperoxides (LH) values over time in NSAID ($n = 36$) and nonuser ($n = 24$) groups. Postexercise values were not significantly different. Values are means \pm SEM.

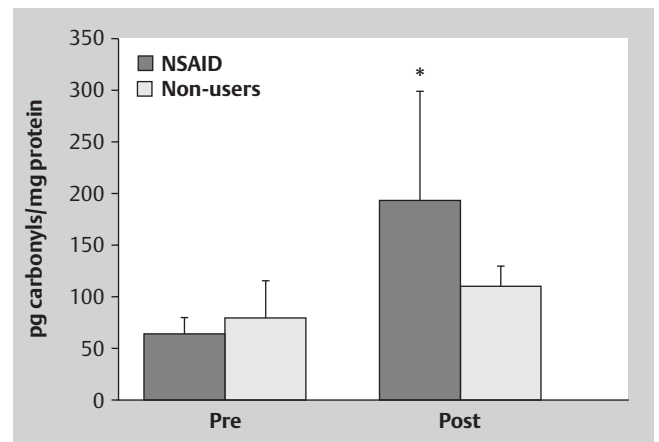


Fig. 6 Protein carbonyls (PC) values over time in NSAID ($n = 43$) and non-user ($n = 17$) groups. * Significantly different from pre-exercise and versus nonusers postexercise. Values are means \pm SEM.

As mentioned previously as a limitation, we only noted individuals who reported use of NSAID during the race. Actual NSAID use was not known or controlled. We have since conducted a controlled NSAID study at the WS-100 ultra endurance race with normal dosages (600 mg) and found significant increases in plasma and urinary F_2 -isoprostanes (unpublished results). It seems probable that our subjects consumed similar amounts. NSAID may induce oxidative stress through bioreductive metabolism to reactive intermediates that have been implicated in oxidative stress and mitochondrial injury [7]. Although not known, we would speculate that individuals who consumed NSAID prior to but not during the race came in with higher oxidative stress markers (F_2 -isoprostanes and PC) but endured less oxidative stress during the race than those who consumed NSAID.

An interesting observation was made in regards to average age of our subjects. Aging is associated with increased free radical generation in the skeletal muscle that can cause oxidative modification of protein, lipid, and DNA [20]. Although there was a significant age difference between NSAID users and nonusers with nonusers being older, oxidative markers were not different with the exception of PC (data not reported). Despite the nonusers being older on average, they experienced less protein oxidation than the younger NSAID users.

We are aware of only one other published study attempting to link muscle damage with oxidative stress following ultra-distance races. Inayama et al. [19] studied the influence of a full marathon race on the status of plasma sulfhydryl groups in moderately trained males and found values were significantly less immediately after the race, 24 h after, and 48 h after the race compared to baseline. Plasma concentrations of CPK were also significantly increased. In the present study, NSAID use did not attenuate plasma CPK levels or post-race DOMS, and there was no correlation between NSAID use, CPK, or DOMS with any oxidative stress measure. As NSAIDs primarily affect the cyclooxygenase system, our data support previously established concepts that F_2 -isoprostanes are mainly derived from free-radical mediated peroxidation of arachidonic acid and not from cyclooxygenase pathways [29]. We have previously observed significant increases in F_2 -isoprostanes as a result of ultra-endurance races [26,30]. Although we observed no association with F_2 -isoprostanes and CPK, Hinchcliff et al. [18] found that plasma F_2 -isoprostanes were positively correlated with plasma creatine kin-

ase activities in sled dogs exposed to consecutive days of constant exercise. The physiological ramifications of the exercise associated increase in F_2 -isoprostanes are not currently known, but research is planned by our group to further examine this oxidative stress component. Several investigators have reported no beneficial effect of NSAIDs in alleviating muscle soreness and damage after contraction-induced muscle injury [2,15]. However, Tokmakidis et al. [35] found that ibuprofen significantly reduced plasma CPK after eccentric leg exercise.

We are unaware of other published studies indicating changes in oxidative stress in NSAID users compared to nonusers following ultramarathons. The relationship between NSAID use and oxidative stress is the potential for endotoxemia to occur. Endotoxemia is caused by increases of bacterial lipopolysaccharide (LPS) infiltrating into the blood from the gut due to increased gastric permeability from NSAID use. Endotoxemia has also been found to occur solely as a result of exercise [1,8]. LPS has been used in animal models to induce oxidative stress [29]. Therefore, use of NSAIDs during ultramarathons may augment oxidative stress by inducing endotoxemia.

We had predicted that NSAID use would possibly diminish inflammation from the immune responses and thereby decrease oxidative stress, but this apparently did not occur [22,36]. F_2 -isoprostanes were significantly increased as a result of the race but, as previously stated, there were no group differences. This indicates that NSAID use or nonuse was not associated with an inflammatory effect on F_2 -isoprostanes in our study. Although not reported here due to the difference in scope, Nieman et al. [31] found that NSAID use was associated with increases in several cytokines.

LH were not affected by exercise or any treatment in this study. LH are highly reactive compounds and are thought to originate primarily from oxidation of omega-3 and omega-6 fatty acids found in lipoproteins [32]. Endurance training is known to enhance endogenous enzymatic antioxidant systems which specifically target hydroperoxide compounds [11,12]. Therefore, it is likely that LH did not accumulate due to the highly trained state of our subjects and consequent increase in activities and expression of these antioxidant defense systems.

In contrast to the other oxidative stress markers examined in this study, PC were dramatically increased in the athletes using NSAID versus nonusers. Limited information is available regard-

ing protein oxidation and oxidative stress in humans *in vivo*. As to an explanation for the different effects on oxidative stress markers used in this study, we can only speculate at this time, but it appears likely that the timing of the sampling in regards to stability of the marker is responsible. That is to say that LH are very quickly degraded compared to PC and F₂-isoprostanes and therefore an increase was not detected. Site of origin of the ROS and the location of the potential macromolecular target may also be important. Another possibility may be the mode of exercise utilized. Bloomer et al. [6] compared oxidative modification of blood proteins (PC), lipids, DNA, and glutathione in the 24 hours following aerobic (cycling) and anaerobic exercise (squatting) using similar muscle groups. It was found that only PC and glutathione were affected by the exercise, with the anaerobic exercise generating more stress. This study illustrates that oxidized biomarkers can be differentially affected. The ramifications of increased oxidative stress in regards to PC concentrations in the plasma may be harmful to health and performance. Modified proteins can lead to loss of catalytic or structural function and subsequent degradation of the modified protein. In certain pathological states, oxidized proteins accumulate in cells and contribute to the disease progression [6,17].

It is of importance that total plasma antioxidant potential has been found to be inversely related to oxidative stress in some disease states [14]. We had predicted that total plasma antioxidant potential as measured by FRAP would be increased by NSAID use due to the previously mentioned hypotheses that NSAID inhibit cylooxygenase pathways and prevent immune activation, thereby inhibiting inflammation, oxidative stress, and consumption of antioxidants [9,36]. However, although FRAP values were significantly increased in both NSAID user and non-user groups, there was no difference between groups. The increase in plasma antioxidant potential is most likely due to increasing uric acid and vitamin C in the blood during exercise [33].

In conclusion, our results indicate that NSAID use during endurance exercise does not alleviate muscle damage or DOMS and actually increases certain oxidative stress markers. Furthermore, oxidative stress and muscle damage do not appear to be related during ultra-distance events. As stated earlier, a limitation of the study was that NSAID use was not controlled, so no recommendations can be made about NSAID dosages that should be avoided. Certainly, in the interest of sports medicine, the relationship between oxidative stress and NSAID use should be examined more closely in future studies. Based upon our findings, we recommend caution in using NSAID during ultra-distance exercise events.

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