

## Is Skeletal Muscle Damaged by the Oxidative Stress Following Anaerobic Exercise?

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We investigated whether the injury of skeletal muscle owing to the action of free radicals and the subsequent oxidative damage to tissues occurred during anaerobic exercise. To estimate injury to skeletal muscle, we determined certain indices of oxidative damage to skeletal muscle; i.e., leukocyte counts, concentrations of hypoxanthine, xanthine, urate, tissue- and serum-type CK-M isoforms, myoglobin, and total antioxidant capacity (TAC) of serum. Blood for these tests was collected at 3 min post-exercise. Post-anaerobic exercise concentrations of lactate were significantly increased from pre-exercise. The neutrophil and lymphocyte counts and alanine concentration were significantly increased by anaerobic exercise, even when the results were corrected

for plasma volume changes; the plasma concentrations of hypoxanthine, urate, and TAC of serum were also significantly increased. The plasma concentration of xanthine was negatively correlated with TAC of serum. The activities of tissue- and serum-type CK-M were significantly increased post-exercise. When the hypoxanthine, urate, TAC of serum, myoglobin, and tissue- and serum-type CK-M were corrected for plasma volume changes, the post-exercise increases were no longer significantly different from the pre-exercise results. We suggest that these latter test results following anaerobic exercise exclude the presence of oxidative damage to skeletal muscle. *J. Clin. Lab. Anal.* 15:239–243, 2001. © 2001 Wiley-Liss, Inc.

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Many blood analytes are changed by exercise. A common source of elevations of creatine kinase (EC 2.7.3.2: CK) and myoglobin is damaged muscle. Lactate, alanine, and hypoxanthine are formed in association with metabolic processes that are accelerated by exercise (1–4). Reports exist of exercise-induced changes in blood cells (5,6). An ongoing program of aerobic exercise that increases the maximum oxygen uptake ( $VO_{2max}$ ) reduces the risk of life-style related diseases, especially coronary artery disease (7). Intense anaerobic exercise often damages skeletal muscle (1,4). Skeletal and cardiac muscle and other organs may experience hypoxia during anaerobic exercise; these then undergo reoxygenation at the cessation of exercise, and such reoxygenation may induce oxidative damage or oxidative stress in skeletal muscle and elsewhere (4). However, humans in actual life often encountered stern works and could not keep away from the anaerobic exercise. Therefore, we naturally wondered if our muscles were so easily harmed by oxidative stress. As a starting hypothesis, we proposed that skeletal muscle exhibits little or no damage during the anaerobic exercise described here. We wanted to confirm this finding with the relevant tests, performed by us.

## MATERIALS AND METHODS

### Subjects

Seven healthy men (ages 27 to 48 years; mean  $\pm$  SD,  $37 \pm 9$  years) and six healthy women (ages 21 to 30 years; mean  $\pm$  SD,  $24 \pm 3$  years) participated in the study. In all cases, informed consent was obtained, and the project was approved by the Protection of Human Subjects Committee of Toho University. All subjects were untrained volunteers and performed the exercise on a treadmill (METS-900, Model-900 metabolic exercise testing system, Vise Medical, Tokyo, Japan) to reach nearly 100% of their maximal oxygen uptake ( $VO_{2max}$ ); exercise was stopped abruptly at this point.  $VO_{2max}$  was estimated from the time interval when the volume ratio of  $O_2$  to  $CO_2$  in the expired gas remained unchanged. The workload was increased by raising the speed of the treadmill;

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this occurred at 7–15 min after starting the exercise. Using evacuated tubes, we collected plain, heparin-, and EDTA-anticoagulated blood specimens from all subjects just before and within 3 min after the end of the treadmill exercise.

### Assays

The plasma lactate concentrations were determined by a direct enzymatic procedures: enzymatic oxidation of lactate by lactate oxidase (EC 1.1.1.27) followed by a peroxidase (EC 1.11.1.7)-coupled colorimetric method from Kyowa Medex Co., Ltd. (Tokyo, Japan) (8). Differential leukocyte counts were determined using a hematology analyzer (Cell-Dyn 3000, Abbott Diagnostics Division, North Chicago, IL). The plasma alanine concentration was measured using a JLC-300V amino acid analyzer (JEOL, Tokyo, Japan). The total antioxidant capacity (TAC) of serum was determined by our previously reported method; it is based on the AAPH [2,2'-azo-bis(2-amidinopropane) dihydrochloride]-induced hemolysis of erythrocytes (9). The TAC of serum or plasma was expressed as mmol/l of Trolox [(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of  $\alpha$ -tocopherol]. Plasma concentrations of hypoxanthine, xanthine, and urate were determined with our HPLC method using a reversed-phase column (10). CK-MM isoforms were assayed by an immunological procedure using a specific antibody against CK-M that contains lysine at the C-terminal residue (CK-IF; Dia-Iatron Co., Tokyo, Japan) (11). The CK activity of serum preincubated with and without antibodies to CK-M was assayed in a Hitachi 7070 analyzer. The residual activity obtained with the antibody was composed of MM1 + (MM2)/2 + (MB2)/2 + MB1 + BB, and the inhibited activity was composed of MM3 + (MM2)/2 + (MB2)/2 (12). In this study, we defined the residual activity as serum-type CK-M, and the inhibited activity as tissue-type CK-M, since serum activities of CK-MB and CK-BB were quite low and CK-MB was reported not to be affected by exercise (13). We determined serum myoglobin by a photometric immunoassay using a LPIA-200 analyzer (Dia-Iatron Co.). The method is based on the agglutination of latex particles and the subsequently formed turbidity (14).

The pre-exercise analytical values were expressed without a correction for plasma volume changes. After exercise, the concentrations or activities of analytes in plasma (or serum) were affected by hemoconcentration owing to water loss during exercise. Unless otherwise stated, also the post-exercise values were expressed without a correction for plasma volume changes. These values are in fact the real blood concentrations and would still be useful for judging the post-exercise-induced changes. We used the pre- and post-exercise hematocrit to calculate the effect of hemoconcentration (15). With this post-exercise correction, all values were, on average, 8% lower. Statistical analysis be-

tween pre- and post-exercise values was performed using the paired *t*-test. Statistical differences were considered to be significant at  $P < 0.05$ .

### RESULTS

As shown in Fig. 1, the post-exercise concentrations of lactate were significantly correlated with the duration of exercise ( $r = 0.613$ ,  $P < 0.05$ ). In 13 of the volunteers (Table 1), 11 showed lactate concentration of  $>4$  mmol/l; two subjects performing aerobic exercise gave lactates of 3.36 and 3.81 mmol/l. The concentrations of lactate that we found in subjects C to M (Table 1) indicated that running on a treadmill led to an anaerobic state. The generally agreed on value for the transition of aerobic to anaerobic exercise is  $>4$  mmol/l (7). The neutrophil and lymphocyte counts were significantly increased ( $P < 0.001$ ) by anaerobic exercise with or without correction for their plasma volume changes (Table 1, volunteers C to M). The same was true for the plasma alanine ( $P < 0.01$ ). These leukocyte counts and alanine concentrations in subjects A and B (Table 1) were also increased.

The concentrations of hypoxanthine, urate, and TAC were increased significantly ( $P < 0.05$ ) by anaerobic exercise (Table 2, volunteers C to M), but these changes were not significant when corrected for the plasma volume changes. We found no significant changes of the plasma xanthine concentrations ( $P > 0.05$ ) by anaerobic exercise when corrected for the plasma volume changes or not. In volunteers who performed anaerobic exercise, activities of tissue- and serum-type CK-M were significantly increased by anaerobic exercise (Table 3). The typical increase in these analytes was 6%–10%. When both the tissue and serum CK-M activities were corrected for plasma volume changes, the increases were not statisti-

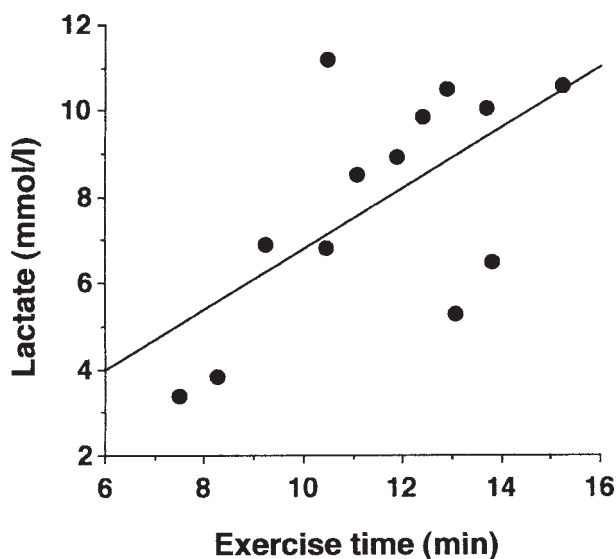


Fig. 1. Correlation of plasma concentrations of lactate to exercise time in 13 volunteers ( $r = 0.613$ ,  $P < 0.05$ ).

**TABLE 1. Leukocyte counts and plasma concentrations of lactate and alanine before and after exercise<sup>a,b</sup>**

	Lactate, mmol/l	Neutrophils, $\times 10^9/l$	Lymphocytes, $\times 10^9/l$	Alanine, $\mu\text{mol/l}$
Volunteers with aerobic exercise				
A; 7.5	0.59 (3.36)	2.4 (2.5)	1.9 (2.1)	327 (436)
B; 8.3	0.57 (3.81)	4.2 (5.6)	2.6 (4.7)	203 (288)
Mean $\pm$ SD	0.58 $\pm$ 0.01 (3.58 $\pm$ 0.32)	3.3 $\pm$ 1.3 (4.1 $\pm$ 2.2)	2.3 $\pm$ 0.5 (3.4 $\pm$ 1.8)	265 $\pm$ 88 (362 $\pm$ 105)
Volunteers with anaerobic exercise				
C; 13.1	0.60 (5.29)	3.2 (7.4)	3.7 (2.5)	274 (427)
D; 13.8	0.60 (6.49)	2.9 (3.6)	2.0 (3.3)	355 (472)
E; 10.5	1.34 (6.80)	3.1 (3.6)	2.0 (4.2)	477 (590)
F; 9.2	0.95 (6.90)	2.2 (3.6)	1.4 (2.8)	363 (390)
G; 11.1	0.77 (8.50)	4.2 (7.9)	1.0 (3.3)	284 (419)
H; 11.9	1.18 (8.93)	3.3 (4.5)	2.2 (6.4)	417 (632)
I; 12.4	0.84 (9.85)	4.4 (4.4)	5.6 (5.6)	382 (491)
J; 13.7	1.07 (10.07)	4.3 (5.7)	2.0 (4.9)	327 (486)
K; 12.9	0.55 (10.49)	1.4 (4.2)	3.0 (7.0)	297 (426)
L; 15.2	1.20 (10.59)	4.4 (5.6)	2.8 (4.7)	375 (453)
M; 10.5	1.29 (11.21)	4.6 (10.9)	2.6 (4.5)	324 (442)
Mean $\pm$ SD	0.94 $\pm$ 0.29 (8.65 $\pm$ 1.99) <sup>c</sup>	3.5 $\pm$ 1.0 (5.6 $\pm$ 2.3) <sup>d</sup>	2.6 $\pm$ 1.3 (4.5 $\pm$ 1.5) <sup>d</sup>	352 $\pm$ 61 (475 $\pm$ 74) <sup>c</sup>

<sup>a</sup>Subjects and exercise duration in minutes are denoted as "A; 7.5," i.e., subject A exercised for 7.5 min on the treadmill. The post-exercise values are in parenthesis and not corrected for plasma volume changes.

<sup>b</sup>Significantly different than the pre-exercise values: <sup>c</sup> $P < 0.001$ ; <sup>d</sup> $P < 0.01$ .

cally significant ( $P > 0.05$ ). We found no significant changes of the serum myoglobin concentrations by anaerobic exercise when corrected for the plasma volume changes or not ( $P > 0.05$ ). Some of the volunteers (D, I, and M) showed remarkably increased post-exercise values; however, the values were still within the reference range (Table 3).

Table 4 shows correlation coefficients of the analyses of the relationship between indices of anaerobic exercise, oxidative stress, and skeletal muscle damage. Although the post-

exercise values were not corrected for plasma volume changes, hypoxanthine and lymphocytes were significantly correlated with plasma lactate ( $P < 0.05$ ). Xanthine was positively correlated with urate ( $P < 0.05$ ) and negatively correlated with TAC of serum ( $P < 0.05$ ). No correlations were detected between indices of skeletal muscle damage (tissue- and serum-type CK-M, and myoglobin) and lactate, alanine, neutrophils, lymphocytes, hypoxanthine, xanthine, urate, or TAC of serum in 13 volunteers.

**TABLE 2. Plasma concentrations of hypoxanthine, xanthine, urate, and total antioxidant capacity of serum before and after exercise<sup>a,b</sup>**

	Hypoxanthine, $\mu\text{mol/l}$	Xanthine, $\mu\text{mol/l}$	Urate, $\mu\text{mol/l}$	TAC, mmol/l
Volunteers with aerobic exercise				
A; 7.5	12.1 (7.1)	2.2 (1.6)	231 (253)	1.47 (1.79)
B; 8.3	12.9 (5.4)	2.4 (1.4)	237 (267)	1.55 (1.75)
Mean $\pm$ SD	12.5 $\pm$ 0.5 (6.2 $\pm$ 1.2)	2.3 $\pm$ 0.1 (1.5 $\pm$ 0.1)	234 $\pm$ 4 (261 $\pm$ 9)	1.51 $\pm$ 0.06 (1.77 $\pm$ 0.02)
Volunteers with anaerobic exercise				
C; 13.1	8.3 (9.9)	2.3 (3.5)	261 (312)	1.29 (1.67)
D; 13.8	6.0 (8.0)	1.5 (1.6)	142 (177)	1.41 (1.59)
E; 10.5	12.6 (15.6)	2.2 (5.2)	424 (406)	0.63 (0.92)
F; 9.2	7.8 (10.3)	2.1 (1.8)	172 (194)	1.30 (1.67)
G; 11.1	10.9 (10.4)	2.1 (2.4)	192 (259)	1.14 (1.14)
H; 11.9	8.8 (9.6)	1.2 (1.3)	317 (331)	2.22 (2.33)
I; 12.4	7.8 (10.6)	3.0 (2.5)	234 (277)	1.55 (1.80)
J; 13.7	9.5 (12.3)	1.9 (1.1)	301 (309)	2.11 (1.79)
K; 12.9	7.6 (10.4)	1.3 (1.3)	226 (252)	1.50 (1.47)
L; 15.2	4.7 (17.6)	2.0 (2.6)	215 (245)	1.19 (1.51)
M; 10.5	4.8 (10.7)	1.2 (3.1)	387 (412)	1.67 (1.75)
Mean $\pm$ SD	8.1 $\pm$ 2.4 (11.4 $\pm$ 2.8) <sup>c</sup>	1.9 $\pm$ 0.5 (2.4 $\pm$ 1.2)	261 $\pm$ 88 (289 $\pm$ 76) <sup>c</sup>	1.46 $\pm$ 0.44 (1.60 $\pm$ 0.37) <sup>c</sup>

<sup>a</sup>Subjects and exercise duration in minutes are denoted as "A; 7.5," i.e., subject A exercised for 7.5 min on the treadmill. The post-exercise values are in parenthesis and not corrected for plasma volume changes.

<sup>b</sup>Significantly different than the pre-exercise values: <sup>c</sup> $P < 0.05$ .

TAC: total antioxidant capacity expressed as Trolox equivalent.

**TABLE 3. Activities of tissue-type CK-M, serum-type CK-M isoforms, and myoglobin concentrations before and after exercise<sup>a,b</sup>**

	Tissue-type CK-M, U/l	Serum-type CK-M, U/l	Myoglobin, µg/l
Volunteers with aerobic exercise			
A; 7.5	49 (48)	77 (78)	15.0 (13.0)
B; 8.3	31 (34)	34 (36)	11.5 (9.5)
Mean ± SD	40 ± 12 (41 ± 10)	53 ± 31 (57 ± 30)	13.2 ± 2.5 (11.3 ± 2.5)
Volunteers with anaerobic exercise			
C; 13.1	32 (32)	48 (51)	9.5 (13.2)
D; 13.8	93 (102)	88 (97)	17.7 (25.3)
E; 10.5	40 (48)	77 (85)	20.8 (18.8)
F; 9.2	37 (65)	58 (65)	10.0 (9.6)
G; 11.1	25 (25)	28 (31)	12.1 (7.9)
H; 11.9	29 (30)	44 (48)	6.4 (5.4)
I; 12.4	50 (56)	68 (78)	17.9 (29.1)
J; 13.7	65 (71)	104 (119)	19.8 (23.2)
K; 12.9	42 (44)	41 (46)	15.0 (16.9)
L; 15.2	26 (28)	50 (51)	13.3 (12.8)
M; 10.5	37 (39)	70 (73)	12.5 (48.1)
Mean ± SD	43 ± 20 (46 ± 23) <sup>c</sup>	62 ± 23 (68 ± 26) <sup>d</sup>	14.1 ± 4.6 (19.1 ± 12.2)

<sup>a</sup>Subjects and exercise duration in minutes are denoted as "A; 7.5," i.e., subject A exercised for 7.5 min on the treadmill. The post-exercise values are in parentheses and not corrected for plasma volume changes.

<sup>b</sup>Significantly different than the pre-exercise values: <sup>c</sup> $P < 0.05$ ; <sup>d</sup> $P < 0.001$ .

## DISCUSSION

We attributed the increase in the plasma alanine concentration to augmented glucose utilization during exercise. Pyruvate is formed during glycolysis as a precursor of lactate and synthesized 2-oxoglutarate and L-alanine in the glucose-alanine cycle. This reaction is catalyzed by alanine aminotransferase (EC 2.6.1.2: ALT). L-Alanine was also formed in the presence of aspartate aminotransferase (EC 2.6.1.1: AST). The AST catalyzed 2-oxoglutarate and L-aspartate to oxaloacetate and L-glutamate. L-Glutamate then serves as a substrate for the ALT reaction. Actually, we found that AST, ALT, and pyruvate were significantly increased in plasma after exercise ( $P < 0.01$ , data not shown). Plasma glucose increased to  $940 \pm 157$  mg/l (corrected for plasma volume changes) or  $1,005 \pm 141$  mg/l (not corrected for

plasma volume changes) from  $778 \pm 161$  mg/l of the pre-exercise value ( $P < 0.01$ ). At normal resting condition, xanthine oxidase exists as a dehydrogenase. This enzyme may be converted to an oxidase by metabolic stress and by neutrophils. During aerobic exercise, the resulting oxidase form uses molecular oxygen instead of NAD as an electron acceptor to catalyze the conversion of hypoxanthine to xanthine and finally to urate (4). Molecular oxygen was thereby reduced, and harmful oxygen radical was formed. However, during anaerobic exercise, oxygen radical are formed during the reoxygenation stage following exercise (16). In our anaerobic exercise, hypoxanthine and urate were significantly increased ( $P < 0.05$ ), therefore oxidative stress occurs. Here, hypoxanthine was formed from ATP through the adenylate kinase (myokinase: EC 2.7.4.3) reaction, and xanthine was rapidly catalyzed to urate.

**TABLE 4. Correlation coefficients between indices of anaerobic exercise, oxidative stress, and skeletal muscle damage<sup>a,b</sup>**

Versus	Myoglobin	Serum-type CK-M	Tissue-type CK-M	TAC	Urate	Xanthine	Hypoxanthine	Lymphocytes	Neutrophils	Alanine
Lactate	0.424 <sup>c</sup>	0.089	-0.055	0.032	0.228	-0.032	0.601 <sup>d</sup>	0.643 <sup>d</sup>	0.378	0.333
Alanine	0.063	0.352	0.161	0.095	0.448 <sup>c</sup>	0.285	0.443 <sup>c</sup>	0.315	0.217	
Neutrophils	0.466 <sup>c</sup>	-0.243	-0.345	-0.032	0.481 <sup>e</sup>	0.235	0.084	-0.032		
Lymphocytes	0.114	-0.122	-0.063	0.247	0.235	-0.232	0.190			
Hypoxanthine	0.100	0.158	-0.167	-0.442 <sup>c</sup>	0.310	0.537				
Xanthine	0.243	0.032	-0.192	-0.626 <sup>d</sup>	0.601 <sup>d</sup>					
Urate	0.418 <sup>c</sup>	0.100	-0.265	-0.063						
TAC	-0.032	0.045	0.032							
Tissue-type CK-M	0.412	0.787 <sup>f</sup>								
Serum-type CK-M	0.513 <sup>c</sup>									

<sup>a</sup>Post-exercise values not corrected for plasma volume changes are compared.

<sup>b</sup>Probability of relationship being significant: <sup>c</sup> $P < 0.20$ ; <sup>d</sup> $P < 0.05$ ; <sup>e</sup> $P < 0.10$ ; <sup>f</sup> $P < 0.01$ .

TAC: total antioxidant capacity.

During the anaerobic metabolic status described above, increased numbers of circulating neutrophils and lymphocytes were found. The increase of neutrophils would be a consequence of recruitment from the marginated pool to ischemic or inflammatory sites (17) in response to nonspecific immune system activation even though the skeletal muscle has not been damaged during exercise of light or moderate intensity (18,19). However, neutrophils contain high activities of enzymes such as myeloperoxidase (EC 1.11.1.7) and NADPH oxidase (EC 1.11.1.2) that may generate oxygen radicals. In addition, neutrophils converted xanthine dehydrogenase (EC 1.2.1.37) to xanthine oxidase (EC 1.2.3.2) (4). Oxygen radicals that are capable of proteolysis injure normal skeletal muscle resulting in the release of these enzymes from cells. Lymphocytes, also recruited from the marginated pool, act to remove damaged cells and eventually reduced their circulating numbers below the pre-exercise values during the recovery stage (1,6,19). We considered whether the skeletal muscle in our volunteers was damaged by oxidative stress during periods of anaerobic exercise. In our study, both circulating numbers of neutrophils and lymphocytes were significantly ( $P < 0.01$ ) increased 3 min after exercise as compared with the pre-exercise levels, and increasing numbers of leukocytes were not correlated with the indices of skeletal muscle damage (tissue- and serum-type CK-M isoforms, and myoglobin). Accordingly, we considered that the tissue damage did not occur during the anaerobic exercise used by us, because the oxidative stress was not great enough to decrease the TAC of serum although the TAC was negatively correlated with the increasing concentration of xanthine (see Table 4). Also, both tissue- and serum-type CK-M corrected for plasma volume changes were not changed significantly at 3 min post-exercise. The absence of significant changes in serum myoglobin concentration also indicated that injury to the skeletal muscle during anaerobic exercise was unlikely.

In conclusion, we measured the indices of oxidative damage to skeletal muscle in anaerobic exercise: leukocytes, hypoxanthine, xanthine, urate, tissue- and serum-type CK-M isoforms, myoglobin, and TAC of serum. A biochemical indication of oxidative damage to skeletal muscle was not observed at 3 min after the cessation of exercise. Anaerobic exercise as carried out by us apparently does not damage skeletal muscle.

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