Differential Effects of Acute and Chronic Exercise on Human Neutrophil Functions

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ABSTRACT

SYU, G.-D., H.-I. CHEN, and C. J. JEN. Differential Effects of Acute and Chronic Exercise on Human Neutrophil Functions. Med. Sci. Sports Exerc., Vol. 44, No. 6, pp. 1021–1027, 2012. Exercise effects on immunity are highly dependent on exercise intensity, duration, and frequency. Purpose: Because neutrophils play an essential role in innate immunity, we investigated whether acute severe exercise (ASE) and chronic moderate exercise (CME) differentially regulate human neutrophil functions. Methods: Thirteen sedentary young males underwent an initial ASE (pedaling on a bicycle ergometer with increasing loads until exhaustion), and they were subsequently divided into exercise (n = 8) and control groups (n = 5). The exercise group underwent 2 months of CME (pedaling on the ergometer at a moderate intensity for 30 min each day) followed by 2 months of detraining. The control group was abstained from regular exercise during these 4 months. Additional ASE paradigms were performed every month (in the exercise group) or every 2 months (in the control group). Neutrophils were isolated from blood specimens drawn at rest and immediately after each ASE for assaying chemotaxis, phagocytosis, citrate synthase activity, and mitochondrial membrane potential (ΔΨm). Additional blood specimens were drawn from the exercise group before and immediately after the first bout of CME to determine the acute moderate exercise (AME) effects on neutrophil functions. Results: The study’s results are the following: 1) the initial ASE enhanced chemotaxis and induced ΔΨm depolarization; 2) AME did not influence any measured parameter in neutrophils; 3) CME increased chemotaxis, phagocytosis, citrate synthase activity, and ΔΨm; 4) the CME effects remained after detraining except phagocytosis; and 5) the ASE effects disappeared after CME and were partially restored after detraining. Conclusions: ASE and CME differentially affected neutrophil functions, whereas AME was ineffective. Moreover, the fact that CME improves neutrophil functions may partially explain why physically active subjects have a low risk of infection. Key Words: TRAINING, DETRAINING, GRANULOCYTE, MITOCHONDRIA, CHEMOTAXIS, PHAGOCYTOSIS

Regular exercise is a practical way to improve immunity. However, the exercise effects on immunity are highly dependent on exercise intensity, duration, and frequency. Acute severe exercise (ASE), marathon running in particular, increases tissue damage and stimulates the secretion of many proinflammatory cytokines (e.g., tumor necrosis factor α and interleukin (IL) 1β) (8,20) and an anti-inflammatory cytokine (IL-6) (22). ASE also increases the risk of upper respiratory tract infection (14,32). In contrast, chronic moderate exercise (CME) lowers many proinflammatory cytokines (e.g., C-reactive protein and IL-1) and elevates an anti-inflammatory cytokine (IL-10) in the circulation (22). As a whole, CME improves immunity as indicated by lowered susceptibility to viral and bacterial infections (14,32).

Because ASE and CME have diverse effects on immune responses, they may differentially regulate neutrophils. Neutrophils, the major cell type responsible for innate immunity, are short-lived leukocytes that undergo spontaneous apoptosis. Our recent studies have shown that ASE accelerates neutrophil apoptosis, whereas CME delays it (24,25). Whether ASE and CME also exert differential effects on neutrophil functions deserves further investigation. When infection occurs, neutrophils rapidly migrate to the infection site (chemotaxis) and ingest the pathogens (phagocytosis). Whereas some studies demonstrate that ASE paradigms enhance neutrophil chemotaxis and phagocytosis (5,7,17), other studies show either suppressive effects or no effect at all (6,23,31). These controversies might be due to the different physical fitness levels of subjects, different exercise protocols, and different assay methods used in various studies. So far, relatively few studies address the effects of CME on neutrophil functions, and self-controlled studies are still lacking.

It is well known that the aerobic capacity in skeletal muscles increases after exercise training. However, whether and how exercise training affects the aerobic capacity in neutrophils remain to be clarified. Neutrophil chemotaxis but not...
phagocytosis or reactive oxygen species (ROS) production is a process dependent on the mitochondrial membrane potential (ΔΨm) (3). Recent human studies have shown that repeated ASE reduces ΔΨm in neutrophils (9,26). Until now, the CME effects on neutrophil aerobic capacity have not been characterized. We thus hypothesized that ASE and CME exerted differential effects on neutrophil functions, possibly due to the diverse changes in ΔΨm. Because our CME paradigm was composed of repeated acute moderate exercise (AME), we further investigated whether CME effects could be (partially) explained by the effects from a single bout of AME or not. In this study, exercise paradigms included ASE, AME, CME, and detraining (DT). Neutrophil functions were evaluated by chemotaxis, phagocytosis, aerobic capacity (indicated by citrate synthase activity), and ΔΨm.

**METHODS**

**Subjects.** The protocol was reviewed and approved by the Human Ethics Committee of the National Cheng Kung University Medical College (Institutional Review Board No. ER-96-92). Written informed consent was received from all participants. The exercise paradigms were modified from our previous reports (28,29). Thirteen healthy sedentary male volunteers between age 20 and 24 yr participated in this study. They fulfilled the following requirements: no regular exercise (less than once per week) in the past 6 months, no smoking, no previous medical record of metabolic or cardiovascular diseases, no recent symptoms of upper respiratory tract infection, and no medication for at least 1 month before the study. All subjects performed the initial ASE. They were then randomly divided into an exercise group (eight subjects) and a sedentary control group (five subjects). There were no significant differences in the anthropometric data between the two groups: age = 22 ± 0 yr versus 22 ± 1 yr, body weight = 62 ± 2 kg versus 66 ± 3 kg, height = 171 ± 2 cm versus 171 ± 3 cm, resting heart rate (HR) = 70 ± 3 versus 65 ± 2 bpm, and maximal HR after the initial ASE = 184 ± 5 versus 184 ± 2 bpm for the exercise and control groups, respectively.

**Exercise paradigms and blood collection.** Subjects underwent various exercise paradigms (for a detailed flow chart, please see Fig. 1). They arrived at 09:00 a.m., rested for about 30 min, and performed the initial ASE on a cycle ergometer (E3200HRT; Vision Fitness, Madison, WI) with continuous increments of workload every 3 min until exhaustion. The HR reached at least 90% of the predicted maximal HR (about 198 bpm) at the end of ASE. Subjects in the exercise group undertook a 2-month CME on a cycle ergometer (30 min d⁻¹, 5 d wk⁻¹ at 60% of maximal workload determined by the initial ASE and adjusted 1 month later) followed by 2-month DT (abstained from regular exercise of any form). During the 4-month period, they received five ASE tests (the ASE durations and maximal workloads were as follows: initial = 31 ± 2 min and 109 ± 7 W, 1-month CME = 40 ± 1 min and 135 ± 4 W, 2-month CME = 48 ± 2 min and 165 ± 7 W, 1-month DT = 44 ± 1 min and 152 ± 5 W, 2-month DT = 42 ± 2 min and 140 ± 5 W). Sedentary control subjects were abstained from regular exercise for 4 months and received three ASE tests (the ASE durations and maximal workloads were as follows: initial = 30 ± 2 min and 104 ± 6 W, 2 months later = 30 ± 3 min and 105 ± 10 W, 4 months later = 29 ± 2 min and 100 ± 7 W). Peripheral venous blood samples were drawn at rest, immediately after the first bout of AME, and immediately after each ASE. Blood specimens were anticoagulated with sodium citrate and stored on ice. Neutrophils collected before exercise were defined as “resting” specimens.

**Neutrophil isolation and culture.** Neutrophils purified by Histopaque density gradient (10771 and 11191; Sigma-Aldrich, St. Louis, MO) were washed in Hanks Balanced Salt Solution (HBSS) and shocked in a 0.2% NaCl hypotonic solution for 30 s to remove contaminating erythrocytes. Part of the specimen was frozen at −80°C for later measurements of the citrate synthase activity. The remainder was resuspended at 5 × 10⁶ cells per milliliter in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum. Neutrophils were used immediately for measuring chemotaxis, phagocytosis, and ΔΨm. The neutrophil purity and viability (>95%, immediately after isolation)
were routinely checked by Wright stain and trypan blue exclusion, respectively.

**Neutrophil chemotaxis and phagocytosis.** Neutrophil chemotaxis was evaluated by using a polycarbonate membrane-free filter membrane (3-μm pore size) sandwiched in a 48-well modified Boyden chamber (2). Freshly isolated neutrophils (5 × 10⁵ per well) were loaded to the migration device containing either forml- methionyl-leucyl-phenylalanine (fMLP; 10⁻⁸ M; Sigma-Aldrich) or fMLP-free buffer in the lower compartment. After 30 min of incubation, the neutrophils that passed through the filter membrane were stained with Diff-Quik (Sysmex, Hyogo, Japan) and counted in five randomly chosen fields. The chemotaxis index was calculated as (fMLP-stimulated migration − random migration) / (random migration). Phagocytosis of fluorescent latex beads (2-μm diameter, nonopsonized; Sigma-Aldrich) was assayed by methods described previously (1). Freshly isolated neutrophils were incubated with a bead suspension (cell/bead ratio = 1:20) for 20 min at 37°C. Cells were subsequently washed, and the phagocytosis function was determined by flow cytometry. Phagocytic neutrophils were defined as those that ingested one or more beads (see Fig. 1B, Supplemental Digital Content, http://links.lww.com/MSS/A131, which demonstrates quantification methods). The phagocytosis index was defined as the ratio between the number of ingested beads and the total number of neutrophils (see Fig. 1C, Supplemental Digital Content, which demonstrates quantification methods; http://links.lww.com/MSS/A131).

**Neutrophil aerobic capacity and ΔΨm.** Citrate synthase activity was assayed by methods described previously (11). Neutrophil proteins were extracted by a protein extraction buffer in the presence of a protease inhibitor cocktail. The citrate synthase activity, reflecting the aerobic metabolic capacity in mitochondria, was determined by 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich) colorimetric changes at 412 nm, which indicated the appearance of coenzyme A with a thiol group. Results were expressed in units of micromole per minute per milligram of protein. Neutrophils with intact ΔΨm were positively stained with Mitotracker Red (250 nM; Molecular Probes, Eugene, OR) at 37°C for 20 min. Cells were subsequently washed, and the percentage of ΔΨm⁺ cells was determined by flow cytometry (see Fig. 1E, Supplemental Digital Content, which demonstrates quantification methods; http://links.lww.com/MSS/A131).

**Pharmacological suppression of neutrophil ΔΨm in vitro.** To clarify the possible role of neutrophil ΔΨm depolarization in neutrophil chemotaxis and phagocytosis, six additional sedentary subjects (age = 21 ± 1 yr, body weight = 67 ± 2 kg, height = 173 ± 1 cm, resting HR = 72 ± 5 bpm) were recruited. Neutrophil ΔΨm could be suppressed by applying cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, an ionophore; Sigma-Aldrich). Freshly isolated neutrophils were incubated with various concentrations of FCCP (0, 10, and 100 nM) for 20 min in the RPMI medium or in the HBSS to reduce neutrophil ΔΨm. Drug-treated neutrophils were then analyzed for chemotaxis, phagocytosis, and ΔΨm as described earlier.

**Statistical analysis.** The Student’s unpaired t-test was used to analyze the differences between the exercise and sedentary control groups. Data from the same subjects were analyzed by paired or repeated analysis. The paired t-test was used to analyze the effects of ASE, AME, or FCCP experiments in the self-controlled studies. One-way ANOVA was used with repeated measures followed by the Bonferroni posttest was used to analyze various time-dependent effects or dose-dependent effects of FCCP treatments. Two-way ANOVA with repeated measures followed by the Bonferroni posttest was used to analyze interactions between ASE effects and CME–DT states. Significant differences were defined as P < 0.05. All data were presented as mean ± SEM, where n was the number of subjects.

**RESULTS**

**Effects of ASE on neutrophil functions in sedentary subjects.** Initially, all subjects (n = 13) underwent an ASE. Neutrophils freshly isolated before and immediately after ASE were analyzed for chemotaxis, phagocytosis, citrate synthase activity, and ΔΨm. ASE significantly augmented chemotaxis and reduced ΔΨm without altering phagocytosis or citrate synthase activity (Fig. 2 and Supplemental Digital Content 2A, http://links.lww.com/MSS/A132).

**FIGURE 2—Effects of ASE on neutrophil functions in sedentary subjects.** At the very beginning, blood specimens were obtained from all subjects both at rest before and immediately after ASE. Neutrophil functional assays were carried out using freshly isolated neutrophils. A. The chemotaxis index, defined as (fMLP-stimulated migration − random migration) / (random migration). B. The phagocytic cell percentage. C. The neutrophil aerobic capacity, indicated by the citrate synthase activity. D. The percentage of cells with intact ΔΨm. Data were analyzed by a paired t-test. *P < 0.05, after ASE versus before ASE; n = 13.
Effects of AME on neutrophil functions in sedentary subjects.

Three days after the initial ASE, subjects in the exercise group underwent their first bout of AME. Neutrophils freshly isolated before and immediately after AME were analyzed for functional changes. AME did not alter any of the following functional parameters: chemotaxis, phagocytosis, citrate synthase activity, and $\Delta \Psi \text{m}$ (Fig. 3 and Supplemental Digital Content 2B, http://links.lww.com/MSS/A132).

Effects of CME and DT on resting neutrophil functions.

Subjects in the exercise group underwent 2 months of CME followed by 2 months of DT. Neutrophils collected under resting conditions were analyzed. Chemotaxis, citrate synthase activity, and $\Delta \Psi \text{m}$ were enhanced after 1 month of CME and remained at high levels after 2 months of DT (Fig. 4 and Supplemental Digital Content 2C; http://links.lww.com/MSS/A132). Phagocytosis, a parameter also enhanced after 1 month of CME, returned to the pre-CME level after 1 month of DT. In comparison, a 4-month sedentary lifestyle did not alter any neutrophil function. Taken together, CME increased neutrophil chemotaxis, phagocytosis, citrate synthase activity, and $\Delta \Psi \text{m}$.

Alterations of ASE effects during the CME and DT periods.

In addition to altering the basal levels of neutrophil functions, CME and DT also influenced the ASE effects. When sedentary subjects exercised regularly, they improved their physical fitness and neutrophil functions (Fig. 4). The ASE-augmented chemotaxis and ASE-suppressed $\Delta \Psi \text{m}$ in the beginning vanished after 1-month CME (Table 1). As expected, when those physically active subjects underwent DT, their physical fitness became deteriorated. However, most of their CME-improved neutrophil functions were sustained (Fig. 4). Moreover, the CME effects on the ASE-altered neutrophil functions were partially reversed after DT, i.e., ASE was able to augment chemotaxis again after...
2 months of DT, but it still could not suppress $\Delta \Psi_m$ even after DT (Table 1).

**DISCUSSION**

This study is the first to show that ASE and CME in sedentary subjects differentially affect the human neutrophil phagocytosis, citrate synthase activity, and $\Delta \Psi_m$. On one hand, the initial ASE showed increased chemotaxis and reduced $\Delta \Psi_m$. On the other hand, CME enhanced neutrophil chemotaxis, phagocytosis, citrate synthase activity, and $\Delta \Psi_m$. Moreover, CME also effectively blunted the ASE-induced neutrophil chemotaxis and $\Delta \Psi_m$ reduction. Our recent study clearly demonstrated that these two exercise paradigms exert opposite effects on neutrophil apoptosis, i.e., ASE accelerates apoptosis, whereas CME retards it (25). Because apoptotic neutrophils are functionally compromised (30), results from these two studies basically support a concept that regular exercise improves the innate immunity (14,32,33).

Neutrophil $\Delta \Psi_m$ reduction likely contributes to some adverse effects of ASE. Repeated ASE impairs human neutrophil $\Delta \Psi_m$ and increases apoptosis (9,26). In our hands, even a single bout of ASE inhibited neutrophil $\Delta \Psi_m$. The process of neutrophil spontaneous apoptosis has been linked to the reduction of $\Delta \Psi_m$ and the increase of mitochondrial permeability (3,12). It was noted that neutrophil mitochondria with low $\Delta \Psi_m$ were fragmented (see Fig. 1D, Supplemental Digital Content, which demonstrates quantification methods; http://links.lww.com/MSS/A131). Mitochondrial fragmentation (fission) facilitates cell apoptosis (4,10). Thus, the $\Delta \Psi_m$ reduction might partially explain why neutrophil functional performance becomes impaired 1 d after ASE (31).

Paradoxically, although ASE suppressed $\Delta \Psi_m$, it augmented chemotaxis (Fig. 2). Chemotaxis is a major neutrophil function that requires intact mitochondria (3). To explore this paradoxical issue, neutrophils isolated from sedentary subjects were exposed to FCCP to suppress $\Delta \Psi_m$ in vitro. Because neutrophils are highly dependent on glycolysis to generate energy and to maintain their $\Delta \Psi_m$ (27), the inhibitory effect of FCCP was only observed under nutrition-free conditions, e.g., in the HBSS but not in the RPMI 1640 medium (see Fig. 3, Supplemental Digital Content, which demonstrates $\Delta \Psi_m$ and neutrophil functions; http://links.lww.com/MSS/A133). We focused on 10-nM FCCP because it induced a $\Delta \Psi_m$ reduction similar to that induced by the initial ASE. However, 10 nM of FCCP in HBSS inhibited neutrophil chemotaxis as well. Therefore, the ASE-augmented chemotaxis might involve other stimulating factors, such as ASE-induced proinflammatory cytokines and stress mediators in the blood (8,17,20,21). The ASE-stimulated neutrophil chemotaxis may counterbalance the suppressive effects of ASE on lymphocytes and natural killer cells and thus prevent the infectious diseases (15,18). However, the ASE-stimulated neutrophil chemotaxis was a transient phenomenon because it returned to basal or lower levels in 24 h (17,31). Moreover, these post-ASE neutrophils showed a shortened lifespan (25).

It is well known that CME increases the aerobic capacity in skeletal muscles. Apparently, CME was capable of promoting the oxidative metabolism in glycolysis-dominating neutrophils as well (Fig. 4). Because neutrophils are relatively short-lived, we speculate that a CME-induced metabolic shift would take place in the bone marrow where neutrophil progenitor cells undergo functional differentiation. In addition, our recent studies showed that CME delays neutrophil apoptosis and enhances the antioxidant reserve (24,25). Because functional compromised mitochondria are a hallmark for the progression of apoptosis in most cells, neutrophils with increased aerobic capacity and $\Delta \Psi_m$ would be expected to function better and survive longer. CME improved neutrophil chemotaxis and phagocytosis. Because neutrophil chemotaxis involves signaling transduction, Ca$^{2+}$ influx, and cytoskeleton rearrangements (13), this process could be linked with the energy state of the cell. Indeed, the CME paradigm increased both $\Delta \Psi_m$ and chemotaxis in neutrophils (Fig. 4), whereas a suppression of neutrophil $\Delta \Psi_m$ by FCCP was accompanied with retarded chemotaxis (see Fig. 3C, Supplemental Digital Content, which demonstrates $\Delta \Psi_m$ and neutrophil functions; http://links.lww.com/MSS/A133). As neutrophil phagocytosis was unrelated to $\Delta \Psi_m$ (see Fig. 3D, Supplemental Digital Content, which demonstrates $\Delta \Psi_m$ and neutrophil functions; http://links.lww.com/MSS/A133), the CME-enhanced neutrophil phagocytosis should be mediated by other mechanisms, such as factors present in the post-CME serum. On one hand, the serum collected from CME subjects or athletes under resting conditions is relatively anti-inflammatory (22). On the other hand, AME (a single bout of moderate exercise) was sufficient to enhance neutrophil phagocytosis via increases in serum Hsp72 level (16). Although CME is usually composed of repeated AME, a single bout of 30-min AME may

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**TABLE 1. Alterations of ASE effects during the CME and DT periods.**

<table>
<thead>
<tr>
<th>After ASE/Resting</th>
<th>Exercise Group (n = 8)</th>
<th>Sedentary Control Group (n = 5)</th>
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<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>1-Month CME</td>
</tr>
<tr>
<td>Chemotaxis index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor (TNF-α)</td>
<td>1.1 ± 0.1*</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Phagocytic* cells (%)</td>
<td>10.2 74.4</td>
<td>10.2 74.4</td>
</tr>
<tr>
<td>Citrate synthase activity (µmol min⁻¹ mg⁻¹ protein)</td>
<td>18.2 74.4</td>
<td>18.2 74.4</td>
</tr>
<tr>
<td>$\Delta \Psi_m$ cells (%)</td>
<td>34.4 74.4</td>
<td>34.4 74.4</td>
</tr>
</tbody>
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The ASE effects (after ASE vs resting) during 4-month periods were analyzed by two-way ANOVA with repeated measures followed by a Bonferroni posttest. The resting values were the same as presented in Figure 4. * P < 0.05, after ASE versus resting.
be insufficient to change any neutrophil functions. Perhaps our moderate exercise time was too short to stimulate neutrophil functional changes because AME paradigms for 45 min ~ 1 h stimulate both chemotaxis and phagocytosis (7,16). It is worth noting that neutrophil functions are impaired after aging (immunosenescence), including decreasing chemotaxis and phagocytosis (19). Although our data showed that the CME improved these parameters in neutrophils in young men, similar mechanisms could contribute, at least in part, to the beneficial effects of regular exercise on immunity in older adults (34).

When CME-trained subjects subsequently underwent DT, their physical fitness became deteriorated. Surprisingly, most of their CME-improved neutrophil functions were sustained more than 2 months after DT (Fig. 4). These long-lasting CME effects on neutrophil functions, i.e., citrate synthase activity, ΔΨm, and chemotaxis, were related to mitochondria. In contrast, the CME effects on neutrophil phagocytosis (a ΔΨm-unrelated function) were relatively short-lived (lasted <1-month DT). Finally, our results clearly showed that CME improved neutrophil functions (Fig. 4) and altered the ASE effects at the same time (Table 1). Therefore, controversial conclusions could be reached from studies applying different exercise protocols to sedentary subjects (5,7,17) or to well-trained subjects (6,23,31). Taken together, results from the current study and our previous study regarding exercise effects on neutrophil apoptosis (25) provide cellular mechanisms explaining the complex interactions between different exercise paradigms and neutrophils and thus shed some light on the exercise effects on innate immunity.

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The authors have no conflict of interest to declare.

The results of the present study do not constitute endorsement by the American College of Sports Medicine.

REFERENCES


