Induction of Lymphocyte Death by Short- and Long-Duration Triathlon Competitions

ADRIANA CRISTINA LEVADA-PIRES1, MARIA FERNANDA CURY-BOAVENTURA2, RENATA GORJÃO1, SANDRO MASSAO HIRABARA2, ENRICO FUINI PUGGINA1, IDICO LUIZ PELLEGRINOTTI3, LUIZ ANTONIO DOMINGUES FILHO1, RUI CURI1, and TANIA CRISTINA PITHON-CURI1

1Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, BRAZIL; 2Post-Graduate Program in Sciences of Human Movement, Institute of Physical Acting Sciences and Sports, Cruzeiro do Sul University, São Paulo, BRAZIL; and 3Methodist University, Piracicaba, São Paulo, BRAZIL.

ABSTRACT

LEVADA-PIRES, A. C., M. F. CURY-BOAVENTURA, R. GORJÃO, S. M. HIRABARA, E. F. PUGGINA, I. L. PELLEGRINOTTI, L. A. DOMINGUES FILHO, R. CURI, and T. C. PITHON-CURI. Induction of Lymphocyte Death by Short- and Long-Duration Triathlon Competitions. Med. Sci. Sports Exerc., Vol. 41, No. 10, pp. 1896–1901, 2009. Purpose: The effect of triathlon competitions on death of lymphocytes from elite athletes was investigated. Material and Methods: Blood was collected from sedentary volunteers and triathletes at rest and after a short-duration triathlon (SDT) and after a long-duration triathlon (LDT—half Ironman) competitions. Results: The athletes had lowered lymphocyte proliferation capacity compared with sedentary volunteers either at rest or after the competitions. There was no difference in the parameters associated with lymphocyte death when sedentary volunteers were compared with triathletes at rest. Lymphocytes from triathletes after SDT competition showed an increase in DNA fragmentation, phosphatidylinerine externalization, and mitochondrial transmembrane depolarization and did not alter membrane integrity when compared with cells from athletes at rest. In contrast, the LDT competition raised the proportion of lymphocytes with loss of membrane integrity when compared with cells from athletes at rest and did not change the apoptotic parameters. The LDT competition induced an increase of reactive oxygen species (ROS) production by lymphocytes compared with triathletes at rest. The SDT competition did not alter ROS production by lymphocytes when compared with cells from triathletes at rest. ROS production by lymphocytes after LDT competition was 60% higher than in SDT. Conclusions: Evidence is presented herein that an LDT competition caused lymphocyte death by necrosis, whereas an SDT induced lymphocyte apoptosis. The mechanism for lymphocyte death induced by the triathlon competitions may involve an increase in ROS production at different extents. Key Words: PHYSICAL EXERCISE, LYMPHOCYTE PROLIFERATION, ROS PRODUCTION, APOPTOSIS, NECROSIS, ELITE ATHLETES

Apoptosis is characterized by cell shrinkage, membrane blebbing, and chromatin condensation. During apoptosis, there is activation of endogenous endonucleases and caspases that results in irreversible DNA fragmentation along with formation of cell membrane-bound apoptotic bodies (10,28). The cells also die by necrosis, which occurs when adenosine triphosphate (ATP) bound apoptotic bodies (10,28). The cells also die by necrosis, whereas an SDT induced lymphocyte apoptosis. The mechanism for lymphocyte death induced by the triathlon competitions was 60% higher than in SDT.

Conclusions: Evidence is presented herein that an LDT competition caused lymphocyte death by necrosis, whereas an SDT induced lymphocyte apoptosis. The mechanism for lymphocyte death induced by the triathlon competitions may involve an increase in ROS production at different extents.

Key Words: PHYSICAL EXERCISE, LYMPHOCYTE PROLIFERATION, ROS PRODUCTION, APOPTOSIS, NECROSIS, ELITE ATHLETES

A

poptosis is characterized by cell shrinkage, membrane blebbing, and chromatin condensation. During apoptosis, there is activation of endogenous endonucleases and caspases that results in irreversible DNA fragmentation along with formation of cell membrane-bound apoptotic bodies (10,28). The cells also die by necrosis, which occurs when adenosine triphosphate (ATP) production ceases abruptly, being characterized by cell swelling, loss of membrane integrity, and inflammation (29).

Exercise can modulate immune function depending on its frequency, duration, and intensity (23). Intense long-duration exercise is associated with an increased risk of infections in the upper respiratory tract (23). This has been thought to be a consequence of postexercise immunosuppression, which is characterized by reduced natural killer cell activity, lymphocyte proliferation (34), neutrophil phagocytosis (12), and leukocyte microbicidal activity (37). High-intensity exercise also accelerates the process of leukocyte death (8). Mars et al. (18) documented for the first time that apoptosis occurs in human lymphocytes after a high-intensity treadmill test to exhaustion. Evidence has been accumulated that the occurrence of lymphocyte apoptosis is closely associated to the exercise intensity (14,19,20). Mooren et al. (19) showed in sedentary volunteers that an exhaustive (80% VO2max) exercise induces apoptosis in peripheral blood lymphocytes, whereas moderate (60% VO2max) exercise does not.

Different responses have been observed in trained and untrained individuals subjected to an aerobic exercise (20). Lymphocyte apoptosis is substantially enhanced in the badly trained athletes after a marathon, whereas it is not observed in the highly trained ones. Peters et al. (27) demonstrated, after trials of long-duration exercise (150 min), the
protective effect on lymphocyte death by a well-trained condition. These observations indicate that the athlete's individual training condition is an important factor in determining the occurrence of lymphocyte death induced by exercise (20,27).

In spite of the information above, lymphocyte function after an intense exercise such as triathlon competition has not been investigated. Triathlon competition comprises swimming, cycling, and running. The International Triathlon Union distinguishes four different distance categories: short, Olympic, half, and Ironman. The short-duration triathlon (SDT) is a category of short-duration, approximately 60 min, which consists of 0.75-km swimming, 20-km cycling, and 5-km running. The half Ironman is a category of long-duration, approximately 4 h, which is composed of 2-km swimming, 80-km cycling, and 20-km running. The aim of this study was to compare the effect of both half Ironman (~4 h) and SDT (~1 h) competitions on lymphocyte death in elite athletes. Lymphocyte death was investigated by measuring the integrity of plasma membrane, DNA fragmentation, phosphatidylethanolamine (PS) externalization, and mitochondrial transmembrane potential (MTP). Lymphocyte proliferation capacity and reactive oxygen species (ROS) production were also investigated.

**MATERIALS AND METHODS**

**Subjects**

The study was approved by the ethical committee of the Institute of Biomedical Sciences, São Paulo University (number 691). All human blood donors signed written informed consent. All subjects had not taken medication for at least 2 wk preceding the experiment.

**Anthropometric and VO₂max Measurements**

Measurements of the total body mass (kg), height (m), and skinfold (Table 1) were performed according to the International Society for the Advancement of Kinanthropometry (36). Aerobic power and aerobic capacity tests were performed on a treadmill (ATL-10.200; Imbramed, Porto Alegre, Brazil) to determine VO₂max of the triathletes. ECG was carried out to detect possible cardiac pathologies during the physical effort. The subjects performed a graded exercise test with increasing oxygen consumption (VO₂max) (Model Vmax 29; SensorMedics, Yorba Linda, CA) with variation uphill and of velocity (Table 1). A similar protocol of exhaustion was used by Heck et al. (7). The characteristics of the triathletes are also shown (Table 1).

**Experimental Design**

**Subjects.** Blood was collected from the following subjects and conditions: 1) 11 sedentary volunteers who did not perform physical activity; 2) 23 male triathletes at rest; 3) 12 athletes who performed a long-duration triathlon (LDT) competition in Úbaba, São Paulo, Brazil; and 4) 11 athletes who performed an SDT competition in Santos, São Paulo, Brazil.

**Blood sample collection.** Blood samples (25 mL) were collected from the antecubital vein of the triathletes at rest and immediately after the competitions. Blood samples from sedentary group were obtained at rest and under similar conditions, those performed after the competition.

**Lymphocyte preparation.** Lymphocytes were isolated from peripheral blood of the sedentary volunteers and triathletes as described by Boyum (2) and in our previous studies (4). Blood was diluted in phosphate-buffered saline (PBS, 1:1, pH 7.4 containing 100 mmol·L⁻¹ CaCl₂ and 50 mmol·L⁻¹ MgCl₂) and carefully layered on Histopaque (d = 1.077). The tubes were then centrifuged at 400g and 4°C for 30 min. Peripheral blood nucleated cells (PBMC; a mixture of monocytes and lymphocytes) were collected from the interface, lysed with 150 mmol·L⁻¹ NH₄Cl, 10 mmol·L⁻¹ NaHCO₃, 0.1 mmol·L⁻¹ EDTA, pH 7.4, and washed once with PBS. The PBMC were maintained in RPMI-1640 medium to allow the adherence of monocytes to the plates as to obtain a pure lymphocyte preparation (approximately 98%). Lymphocytes obtained from the sedentary volunteers and triathletes were counted in a Neubauer chamber under an optical microscope (Nikon, Melville, NY).

**Lymphocyte proliferation assay.** Proliferation of lymphocytes was determined by the incorporation of [2-¹⁴C]-thymidine into DNA. Lymphocytes were cultured at a density of 2.5 × 10⁵ cells per well in 96-well plates. Cells were stimulated with 5 µg concanavalin A (ConA)·mL⁻¹, a T-lymphocyte mitogen (26). The plates were incubated in a humidified atmosphere with 5% CO₂ and 95% air at 37°C. After 48 h, [2-¹⁴C]-thymidine (1 µCi·mL⁻¹) was added to the medium, and the cells were incubated for a further period of 18 h. The radioactivity was analyzed using a scintillation counter (TRI-CARB 2100 TR counters; Packard, Downers Grove, IL).

**Cell viability assay.** Lymphocytes (1 × 10⁶ cells per milliliter) were resuspended in 500 µL of PBS and 50 µL of propidium iodide (PI) solution (20 µg·mL⁻¹ in PBS) were then added. The percentage of viable cells in each sample was determined by using a FACS Calibur flow cytometer (Becton Dickinson, San Juan, CA). Propidium iodide is a highly water-soluble fluorescent compound that cannot pass through intact membranes and is generally

| Table 1. Anthropometric data and VO₂max from male sedentary volunteers and triathletes. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Group**       | **Age (yr)**    | **Mass (kg)**   | **Height (m)**  | **Body Fat (%)**| **VO₂max**      |
| Sedentary       | 31.6 ± 2.3      | 74.4 ± 2.8      | 1.74 ± 0.02     | 20.5 ± 2.1       | —               |
| Triathletes     | 31.0 ± 1.8      | 79.4 ± 3.1      | 1.77 ± 0.01     | 12.0 ± 1.4       | 63.5 ± 3.85     |
| *(n = 11)*       | *(n = 23)*       |                 |                 |                 |                 |

The values are presented as mean ± SEM.

* P < 0.05 compared with the sedentary group.
excluded from viable cells. It binds to DNA by intercalating between the bases with little or no sequence preference. Fluorescence was measured using the FL2 channel (orange-red fluorescence = 585/42 nm). Ten thousand events were analyzed per experiment. Cells with PI fluorescence were then evaluated by using the Cell Quest software (Becton Dickinson). Similar measurement was performed in our previous studies (4,16).

**DNA fragmentation measurement.** DNA fragmentation was analyzed by flow cytometry after DNA staining with PI according to the method described by Nicoletti et al. (22) and in our studies (14,16). Lymphocytes (2 × 10^6) were gently resuspended in 300 µL of hypotonic solution containing 50 µg·mL^{-1} PI, 0.1% sodium citrate, and 0.1% Triton X-100. The cells were then incubated for 30 min at 4°C. The presence of detergent in the solution permeabilizes the cells, which promptly incorporates the dye into the DNA. Fluorescence was measured and analyzed as described above.

**PS externalization.** PS externalization was analyzed by flow cytometry after PS staining with fluorescein-conjugated Annexin V (Annexin V–FITC). PI is used to distinguish viable from non-viable cells. Fluorescence of Annexin V–FITC was measured in the FL1 channel (green fluorescence = 530/30 nm) and PI in FL2 channel (orange-red fluorescence = 585/42 nm). Cells stained with Annexin V–FITC were then evaluated as described above. Similar measurement was performed in our previous studies (4,16).

**MTP.** Lymphocytes (1 × 10^6) were incubated for 15 min at 37°C with rhodamine 123 (5 µmol·L^{-1}) in the dark. Afterward, the cells were washed twice with cold PBS, incubated for 30 min at 30°C in the dark and analyzed in a flow cytometer as described above. Rhodamine 123 is a cell-permeable, cationic, fluorescent dye that is readily sequestered by active mitochondria without inducing cytotoxic effects. Uptake and equilibration of rhodamine 123 is rapid. Therefore, rhodamine 123 allows for a quick and easy detection of changes in MTP. Fluorescence of rhodamine 123 was determined using the FL1 channel (green fluorescence = 530/30 nm), as reported in our previous study (16).

**Production of ROS.** Dihydroethidium was used for the flow cytometric measurement of ROS. Dihydroethidium is rapidly oxidized to ethidium bromide (a red fluorescent compound) (35). Ethidium is trapped in the nucleus by intercalating into the bases of DNA, leading to an increase in ethidium fluorescence. Lymphocytes (1 × 10^6 cells per milliliter) were stained with dihydroethidium (1 µmol·L^{-1}) and incubated for 30 min at room temperature in the dark and analyzed in a flow cytometer. Fluorescence was measured in the FL3 channel (670 nm). Histograms of 10,000 events were analyzed per assay as previously reported (4,16).

**Statistical Analysis**

Results are presented as means ± SEM. Differences were assessed by ANOVA and Tukey-Kramer multiple comparison test using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The significance level was set for P < 0.05.

**RESULTS**

The training for both triathlon competitions reduced lymphocyte proliferative capacity. Even before the competition, ConA-stimulated lymphocyte proliferation was reduced in the triathletes by 59% compared with sedentary volunteers. LDT and SDT competitions decreased ConA-stimulated lymphocyte proliferation by 41% and 61%, respectively, when compared with the sedentary group (Figs. 1A and B). Both triathlon competitions did not change lymphocyte proliferation compared with rest condition. Proliferation of lymphocytes in the absence of ConA was not affected by training and triathlon competitions.

The LDT competition increased the proportion of lymphocytes with loss of membrane integrity by 22% when compared with the cells from triathletes at rest. The SDT did not change lymphocyte membrane integrity (Fig. 2A). In contrast, there was a 2.2-fold increase in the proportion of lymphocytes with fragmented DNA after the SDT.
compared with triathletes at rest (Fig. 2B). The LDT 
competition did not alter the proportion of lymphocytes 
with PS externalization. However, the SDT competition 
increased by 2.9-fold the proportion of cells with PS 
externalization compared with triathletes at rest (Fig. 2C).
The LDT competition did not alter MTP, whereas the SDT 
caused a 2.4-fold increase compared with lymphocytes from 
triathletes at rest (Fig. 2D). There was no difference in the 
parameters of cell death when sedentary volunteers were 
compared with triathletes at rest (Fig. 2).

Changes in ROS production by lymphocytes were also 
different in the two triathlon competitions. ROS production 
was higher in lymphocytes from triathletes at rest (by 8-fold) 
and after LDT (by 13.5-fold) and SDT (by 8.5-fold) 
competitions compared with sedentary volunteers. The 
LDT competition induced an increase of 1.6-fold in ROS 
production by lymphocytes compared with triathletes at rest. 
However, SDT did not alter ROS production by lymphocytes 
when compared with cells from triathletes at rest. ROS 
production by lymphocytes after LDT competition was 60% 
higher than that in SDT (Fig. 3).

DISCUSSION

Lymphocyte proliferation was reduced in triathletes at rest 
and after both triathlon competitions, suggesting that this 
function is altered by the exercise training itself and/or may 
be associated to an increased muscle mass. This change may 
account for the increased occurrence of upper respiratory 
infections in trained athletes (23). The reduction of 
lymphocyte proliferation capacity by physical exercise has 
also been found by others in the recovery period after a 
physical exercise session in treadmill, running, and cycling 
and a marathon (5,17).

Several studies have shown that high-intensity exercise 
increases the percentage of apoptotic lymphocytes in 
untrained individuals (8,18,19,24). The high occurrence of
lymphocyte apoptosis has been associated with postexercise immunosuppression (18). In fact, a decrease in lymphocyte number, below baseline values, is observed after prolonged exercise (27,38). The possibility that the decrease in the number of lymphocytes may reflect an increase in lymphocyte death was investigated. Evidence is presented herein that the duration of a high-intensity exercise promotes lymphocyte death in elite athletes by different mechanisms. The increase in lymphocyte apoptosis observed after the SDT competition corroborates the findings by others (4,8,18,19,24). Apoptosis of lymphocytes was assessed by the increase in DNA fragmentation, depolarization of mitochondrial membrane, and increase in PS externalization. In contrast, LDT competition, performed by well-trained athlete, did not induce lymphocyte apoptosis as indicated by the results of DNA fragmentation and mitochondrial membrane depolarization. Peters et al. (27) also did not find DNA damage or PS externalization in lymphocytes from well-trained endurance athletes after a 2.5-h trial. The same was observed by Mooren et al. (20) in well-trained athletes after a marathon competition. In our study, there was an increase in the proportion of cells with loss of membrane integrity after the LDT competition, suggesting that the cells are dying by necrosis. Peters et al. (27) and Mooren et al. (20) did not examine plasma membrane integrity.

High-intensity exercise has been shown to cause apoptosis of leukocytes as indicated by various measurements: mitochondrial membrane depolarization (4,8,16), PS externalization (14,16,20), reduction of DNA content (40), increased levels of cytochrome c and caspase 3 protein expression in the cytosol, increased expression of proapoptotic Bax and Bcl-Xs gene (14,16,31), decreased antiapoptotic Bcl-2 protein levels and Bcl-XI gene expression (14,16,31), and chromatin condensation (14). However, this is the first evidence that an LDT competition induces lymphocyte death by necrosis. In a previous study, we demonstrated that an LDT competition (half Ironman) induced neutrophil apoptosis (16). So, the effect of physical exercise duration on leukocyte death varies also with the cell type.

The mechanisms of cell death induced by high-intensity exercise seem to involve Ca\(^{2+}\) release (1) and increased ROS production (38). ROS interact with other molecules damaging various cellular components. Mitochondrial depolarization can potentially be triggered by oxidative stress (3). After SDT competition, an increase in mitochondrial membrane depolarization was observed, suggesting that lymphocyte death was induced by the intrinsic apoptotic pathway (13). During mitochondrial dysfunction, cytochrome c is released to the cytosol that is closely associated with a decrease in ATP synthesis. Changes in the ATP/adenosine diphosphate ratio determine the type of cell death. A moderate decrease of ATP levels leads to apoptosis, whereas a marked decrease causes necrosis (13). The cytochrome c leakage also precedes activation of caspases that leads to degradation of target proteins and is involved in events such as DNA fragmentation (13,24). So, in the SDT competition, lymphocyte death was probably induced by the change in mitochondrial function.

The exposure to high levels of ROS induces necrosis, whereas low levels have been shown to trigger apoptosis (9,15). This fact may explain the differences in lymphocyte death observed after SDT and LDT competition. ROS production was greater in lymphocytes after the LDT competition. In fact, antioxidant supplementation with N-acetyl-l-cysteine and vitamin E prevents lymphocyte apoptosis after an exhaustive exercise (6,33). In our study, ROS production was increased in lymphocytes from triathletes at rest and after both triathlon competitions, indicating that the training can alter this function as also the competition itself.

In spite of the increase in ROS production, lymphocyte death was not observed in triathletes at rest. This fact may be attributed to the glutathione content (GSH) as reported by Wang and Huang (40). Heavy exercise diminishes GSH content and subsequently enhances the oxidative stress-induced apoptosis of lymphocytes and thymocytes (30,32,40). Moderate exercise attenuates lymphocyte apoptosis induced by oxidative stress possibly by enhancing intracellular antioxidant activity (40). In fact, moderate exercise has been associated with an improvement in exogenous antioxidant defense (39). At rest, athletes involved in Ironman triathlon training show a significant decrease in plasma malondialdehyde concentration and increase in activities of glutathione peroxidase and catalase in erythrocyte (11). However, a triathlon competition induces temporary increase in most oxidative stress markers (11,16,21), and reduces the activities of glutathione peroxidase, superoxide dismutase, and catalase in erythrocytes (21).

Recently, our group demonstrated that diet supplementation with hydrolyzed whey protein enriched with glutamine dipeptide (700 mg) protected lymphocytes against apoptosis after a single bout of exercise in elite triathletes (4). One product of glutamine and glutamate metabolism is GSH, which stabilizes neutrophil mitochondrial function and so delays apoptosis (25).

In conclusion, evidence is presented herein that an LDT causes lymphocyte death by necrosis, as shown by the loss of membrane integrity. On the other hand, the SDT induced lymphocyte apoptosis, as indicated by increased DNA fragmentation, plasma membrane PS externalization, and depolarization of mitochondrial membrane. The substantial increase in ROS production by lymphocytes from LD triathletes may be related to lymphocyte death by necrosis, whereas the modest increase of ROS production in lymphocytes from SD triathletes led to apoptosis.

The authors are indebted to the technical assistance of J.R. Mendonça, G. de Souza, E. Portiolli, and T.C. Alba-Loureiro. This research is supported by CNPq, FAPESP, and CAPES. The results of the present study do not constitute endorsement by ACSM.
REFERENCES


