Mild endotoxemia, NF-κB translocation, and cytokine increase during exertional heat stress in trained and untrained individuals

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Selkirk GA, McLellan TM, Wright HE, Rhind SG. Mild endotoxemia, NF-κB translocation, and cytokine increase during exertional heat stress in trained and untrained individuals. *Am J Physiol Regul Integr Comp Physiol* 295: R611–R623, 2008. First published June 18, 2008; doi:10.1152/ajpregu.00917.2007.—This study examined endotoxin-mediated cytokinemia during exertional heat stress (EHS). Subjects were divided into trained [TR; $n = 12$, peak aerobic power ($\dot{V}O_{2peak}$) = 70 ± 2 ml·kg lean body mass^{-1}·min^{-1}] and untrained (UT; $n = 11$, $\dot{V}O_{2peak}$ = 50 ± 1 ml·kg lean body mass^{-1}·min^{-1}) groups before walking at 4.5 km/h with 2% elevation in a climatic chamber (40°C, 30% relative humidity) wearing protective clothing until exhaustion (Exh). Venous blood samples at baseline and 0.5°C rectal temperature increments (38.0, 38.5, 39.0, 39.5, and 40.0°C/Exh) were analyzed for endotoxin, lipopolysaccharide binding protein, circulating cytokines, and intranuclear NF-κB translocation. Baseline and Exh samples were also stimulated with LPS (100 ng/ml) and cultured in vitro in a 37°C water bath for 30 min. Phenotypic determination of natural killer cell frequency was also determined. Enhanced blood (104 ± 6 vs. 84 ± 3 ml/kg) and plasma volumes (64 ± 4 vs. 51 ± 2 ml/kg) were observed in TR compared with UT subjects. EHS produced an increased concentration of circulating endotoxin in both TR (8 ± 2 pg/ml) and UT subjects (15 ± 3 pg/ml) (range: not detected to 32 pg/ml), corresponding with NF-κB translocation and cytokine increases in both groups. In addition, circulating levels of tumor necrosis factor-α and IL-6 were also elevated combined with concomitant increases in IL-1 receptor antagonist in both groups and IL-10 in TR subjects only. Findings suggest that the threshold for endotoxin leakage and inflammatory activation during EHS occurs at a lower temperature in UT compared with TR subjects and support the endotoxin translocation hypothesis of exertional heat stroke, linking endotoxin tolerance and heat tolerance.

splanchnic permeability; immune function; blood volume; cardiovascular/thermoregulatory strain; flow cytometry

EXERTIONAL HEAT STRESS (EHS) produces cardiovascular and thermoregulatory strain due to competition for maintenance of an adequate blood supply to the periphery to promote heat loss and to deliver nutrients to metabolically active muscle (30). Circulatory stability is maintained by the redistribution of cardiac output, accomplished by vasoconstriction of renal and splanchnic vasculature reducing gastrointestinal blood flow (57–59, 70, 71). The redistribution of cardiac output away from abdominal organs to active skeletal muscle during exercise has been correlated to oxygen consumption and is proportional to the increase in heart rate (HR) (70). The extent of the reduction in splanchnic blood flow depends on individual maximum aerobic power but can be reduced by as much as 80%, resulting in intestinal ischemia (58, 70). Exercise, when combined with hyperthermia, can have an additive effect that exasperates the reduction in visceral blood supply, intestinal ischemia, and permeability of the gut wall (30, 70).

Endotoxemia plays a pivotal role in the pathophysiology of the systemic inflammatory response syndrome (SIRS) in various conditions, including sepsis (37, 42), trauma (21), and exertional heat stroke (4, 39, 40). Regulation of these inflammatory disorders is accomplished primarily through NF-κB, a central mediator of inflammatory gene transcription, which can have effects on both innate and adaptive immunity (42).

Endotoxin, or LPS, is normally found in large abundance on the surface of gram-negative bacteria in the intestinal lumen (72). Under physiological conditions, endotoxins are contained and not toxic (76), since only small amounts enter the circulation from the intestinal lumen and are quickly inactivated by several defense mechanisms (20). However, failure of the body's defense mechanisms and/or barriers results in substantial endotoxin leakage across the intestinal lumen into the portal and, eventually, the systemic circulation. Lipopolysaccharide binding protein (LBP) forms an LPS-LBP complex enhancing the transfer of LPS to its receptor complex [CD14/Toll-like receptor (TLR)-4/MD-2] (21, 37) on immune-competent cells (75), resulting in NF-κB activation and translocation from the cytoplasm to the nucleus. NF-κB DNA binding in the liver (14), intestinal epithelial/mucosal cells (63), and, finally, circulating leukocytes (42) activates κB genes encoding inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and inducible nitric oxide synthase (iNOS) in addition to acute phase proteins, leading to activation of SIRS, perfusion abnormalities, intravascular coagulopathy, and ultimately, multiple organ dysfunction syndrome (MODS) (14).

Examination of exertional heat stroke victims has shown an inflammatory profile similar to that seen during endotoxic shock (4, 5, 72). The similarities in pathogenesis between conditions have contributed to the acceptance that exertional heat illness (EHI) is driven in part by an endotoxin-mediated systemic inflammatory response (39, 40, 79). Indeed, endotoxemia is a key factor in the progression of SIRS during exertional heat stroke at core temperatures above 41°C (4); however, there is limited data demonstrating endotoxin-mediated cytokinemia at lower temperatures. It is well documented that strenuous exercise is accompanied by an increase in gastrointestinal distress (33, 40). Many studies also have shown subclinical increases in circulating proinflammatory cytokines during strenuous exercise (84) and exertional hyperthermia (60, 67, 81); yet, those that have documented mild endotox-

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emia failed to report changes in body temperature levels (9, 33). Studies that have controlled the increase in thermal strain have shown a significant reduction in proinflammatory activation in neutral compared with hyperthermic environments (60, 67, 81).

The advantages of regular aerobic exercise during uncompensable EHS are widely documented. A primary mechanism conferring improved heat tolerance in aerobically fit individuals during uncompensable EHS is the ability to tolerate a higher rectal temperature (T_{re}) at exhaustion (Exh) (77). Traditionally, it has been suggested that tolerance to higher levels of T_{re} is due to an increased cardiovascular/thermoregulatory stability. It is not known, however, whether improved cardiovascular stability associated with aerobic training impacts endotoxin translocation, inflammatory activation, and subsequent heat tolerance. Reducing endotoxemia has been found to improve heat tolerance in animals (25), and recent findings also have linked regular aerobic exercise to improved endotoxin tolerance (16, 22, 81), possibly through the enhanced induction of heat shock protein 72 (HSP72) and reduced NF-κB activation (13, 64).

Building on the concept of "heat sepsis" (40), we hypothesized that enhanced cardiovascular strain, leading to the redistribution of splanchnic blood flow away from the gut, will result in increased inflammatory activation in sedentary-untrained compared with endurance-trained individuals at a given level of thermal strain. To test this hypothesis, we administered an uncompensable EHS model to sedentary untrained and endurance-trained individuals, with markers of endotoxin translocation and inflammatory activation measured at specific increments in absolute thermal load. Thus, the purpose of the present study was to examine the progression of endotoxin leakage, inflammatory activation, and the resultant circulating cytokine profiles at different levels of thermal strain in an attempt to explain the mechanism(s) associated with the enhanced heat tolerance observed in endurance-trained individuals.

METHODS

Subjects. After the study protocols were approval by the Defence Research and Development Canada (DRDC)-Toronto and York University Human Research Ethics Committees, 23 healthy men were recruited from surrounding universities and running clubs in the greater Toronto area. All subjects were medically screened, and a full explanation of procedures, discomforts, and risks were given before written informed consent was obtained. Potential subjects were excluded if they did not fit the grouping criteria described below or if they were taking any medications. In addition, subjects were screened for a history of allergy to iodides and/or sensitivities to penicillin and sulfa drugs, a contraindication for indocyanine green (ICG) dye injection. All testing was performed in an exercise laboratory or a climatic chamber at DRDC-Toronto.

Baseline measurements. Peak aerobic power ($\dot{V}_{O_{2peak}}$) was measured using open-circuit spirometry on a motorized treadmill. $\dot{V}_{O_{2peak}}$ was defined as the highest observed 30-s value for oxygen consumption ($\dot{V}_{O_2}$) together with a respiratory exchange ratio ≥ 1.15, and the highest value recorded at the end of the exercise test was defined as peak heart rate (HR_{peak}). Body fortress was calculated using the Siri equation (77), which incorporated body density determined using underwater weighing, and residual lung volume determined using body plethysmography. Lean body mass (LBM) was calculated by subtracting body fat from the total body mass.

Blood volume determination. In a separate session, plasma volume was measured using a standard laboratory dilution technique employing ICG (Akron, Buffalo Grove, IL) (48). To avoid interference from hyperlipidemic samples, subjects arrived in a fasted state. Venous flow was occluded for 2 min above systolic blood pressure proximal to the peripheral injection site. Immediately after the release of pressure, ICG was injected (0.25 mg/kg body wt) through an indwelling venous catheter, followed by a 2-ml saline chaser. Blood was sampled from a second venous catheter in the opposite arm, at 1-min intervals (2 to 11) after ICG injection into heparinized vacutainers. Individual calibration curves were determined for each subject using baseline whole blood spiked with known ICG concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mg/l). Optical density of the plasma was read at 805 nm, and a five-point standard curve was constructed. Unknown dye concentrations were determined on the basis of a natural logarithmic plot vs. time and extrapolation using a linear best-fit regression to determine initial dye concentration. Plasma volume and total blood volume were calculated using venous hematocrit and a correction factor of 0.96 (48).

Grouping criteria. Subjects were divided into two groups of endurance-trained (TR; $n = 12$) or untrained individuals (UT; $n = 11$) based on $\dot{V}_{O_{2peak}}$ values expressed relative to LBM and activity profiles (77). TR subjects were defined as actively participating in a cardiovascular training program more than three times per week and having a $\dot{V}_{O_{2peak}} > 65$ ml·kg LBM^{-1}·min^{-1}. UT subjects were defined as being minimally active (<2 times per week) and having a $\dot{V}_{O_{2peak}} < 50$ ml·kg LBM^{-1}·min^{-1}.

Experimental design. All subjects participated in both a familiarization and experimental EHS session, which began at ~8:00 AM. Familiarization sessions involved exposure to all dressing procedures (including venous catheter and rectal probe insertion) as well as a brief heat exposure while walking in the climatic chamber. Each familiarization session was performed at least 10 days before the experimental trial to limit the acute effects of heat acclimation. In addition, heat exposure was limited to 30 min to maintain a T_{re} below 38.0°C, since it has been shown in previous work that the HSP response is not manifested at T_{re} values below 38.0°C (66). Subjects refrained from strenuous exercise (running, swimming, cycling, and weight lifting, among others), alcohol, and the use of nonsteroidal anti-inflammatory drugs for 24 h, and the use of caffeine for 8 h, before each session.

EHS model. During the familiarization session and EHS trial, subjects walked on a motorized treadmill (4.5 km/h, 2% incline, wind speed <0.1 m/s) at 40°C, with 30% relative humidity (Fig. 1). The intensity of exercise was selected such that the elevated metabolic rate, together with the clothing ensemble (described below), created an uncompensable EHS condition and produced exposure times of ~2 and 3 h for UT and TR, respectively. Exhaustion (Exh), which was defined by specific end-point criteria for the experimental heat stress trials, included an ethical T_{re} cutoff of 40.0°C, HR reaching or exceeding 95% of maximum for 3 consecutive minutes, Exh/thermal discomfort, dizziness or nausea precluding further participation, and/or subject or experimenter termination. Tolerance time (TT) was defined as the elapsed time from the beginning of walking on the treadmill to the attainment of one or more of the end-point criteria that resulted in termination of the EHS trial.

Clothing ensemble. The Canadian military nuclear, biological, and chemical protective semipermeable overgarment was worn during familiarization and experimental trials. In addition, combat pants and shirt, underwear, shorts, T-shirt, and running shoes were worn beneath the semipermeable overgarment. No respirator, gloves, or overboots were worn, but the hood was placed over the head. The total thermal resistance of this protective ensemble has been reported previously (77), but it should be noted that these values will be slightly lower in the current study due to the absence of impermeable gloves, boots, and a respirator.
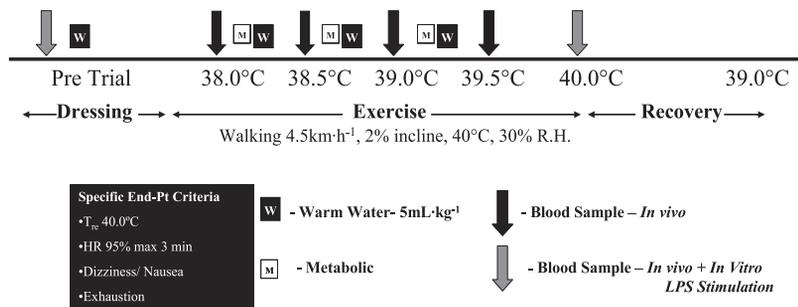
Fig. 1. Protocol timeline for exertional heat stress (EHS) trial at 40°C and 30% relative humidity (RH), with subjects wearing nuclear, biological, and chemical semipermeable overgarments and walking at 4.5 km/h at a 2% incline. T_{re}, rectal temperature; HR, heart rate.

Physiological measurements. HR was monitored using a transmitter (Polar Vantage XL; Polar Electro, Kempele, Finland) attached with an elasticized belt fitted around the chest and taped in place. The receiver was taped to the outside of the clothing, allowing for a continuous HR display. Open-circuit spirometry was used to determine expired minute ventilation, aerobic power ($\dot{V}O_2$), and carbon dioxide production at each T_{re} increment from values averaged over a 2-min sampling period (Fig. 1). T_{re} was measured using a flexible vinyl-covered rectal thermistor (YSI Precisions 4400 Series; Yellow Springs Instruments, Yellow Springs, OH) inserted ~15 cm beyond the anal sphincter. Mean skin temperature (M_{sk}) was obtained from seven temperature thermistors (Mallinckrodt Medical, St. Louis, MO) taped to the head, abdomen, medial deltoid, hand, anterior thigh, shin, and foot, respectively. Mean values over 1-min periods for T_{re} and a seven-point weighted M_{sk} were calculated as described previously (77), recorded, and printed by the computerized data acquisition system. Subjects received 5 ml/kg LBM of warm water (37°C) before entering the climatic chamber and, following metabolic measurements, approximately every 30 min (Fig. 1) during the trial to limit heat-sink effects, reduce circulatory instability produced by progressive dehydration, and increase the level of T_{re} tolerated at Exh. Sweat rate (SR) was calculated using values for nude masses pre- and postexposure, fluid administered, and TT; SR values were corrected for respiratory and metabolic mass losses as described previously (77). Hematocrit and hemoglobin values were determined using a hematology analyzer (Coulter Aᶜ·T diff 2; Beckman Coulter, Miami, FL). Changes in blood and plasma volume were calculated using equations from Dill and Costill (19). Osmolality was calculated using an Advanced Micro-Osmometer (model 3300; Advanced Instruments, Norwood, MA).

Blood collection and storage. Venous whole blood was collected at six sampling times during the experimental heat stress trial by using an indwelling venous catheter and a 24-inch extension that protruded from the sleeve of the protective ensemble. After catheter insertion, subjects remained standing for 20 min to obtain postural stability before the baseline sample was taken. Subsequent samples were taken during the experimental heat stress trial at specific T_{re} intervals (38.0, 38.5, 39.0, 39.5, and 40°C/Exh) rather than at specific time periods (Fig. 1). Catheter patency was maintained by injecting 3 ml of a sterile saline solution between sampling intervals. Blood samples were drawn into sterile syringes and immediately transferred into corresponding vacutainers (BD Biosciences, Franklin Lakes, NJ). Vacutainers containing sodium heparin were used for flow cytometry analyses, and K_2EDTA was used for hematology analyses. K_2EDTA plasma samples for endotoxin determination were always collected first under strict aseptic techniques with certified endotoxin-free tips and storage containers (Associates of Cape Cod, East Falmouth, MA). The remaining supernatants were then transferred into polypropylene Eppendorf tubes. Samples were frozen and stored at −70°C until assayed. All circulating concentrations were corrected for changes in plasma volume (19).

Chromogenic Limulus amebocyte lysate assay. Circulating plasma levels of endotoxin were measured using a *Limulus* amebocyte lysate chromogenic assay (LAL; Associates of Cape Cod) according to the

manufacturers' instructions. Standard endotoxin [*Escherichia coli* O113:H10; mean potency 9.1 endotoxin units (EU)/ng] was reconstituted in pyrogen-free water (PFW) with 1:10 serial dilutions producing final standard concentrations of 50, 5, 0.5, 0.05, and 0.005 EU/ml. Samples were immediately thawed and diluted (1:5) with PFW and heat treated (65°C for 10 min) to remove endotoxin inhibitors (55). Pyrochrome lysate was reconstituted with a 1,3-β-D-glucan-inhibiting buffer to render the assay endotoxin specific. Each sample was assayed in duplicate with corresponding positive control, and an additional 1:2 dilution of each sample was performed on the 96-well plate. Maximum sensitivity of the chromogenic assay was 0.005 EU/ml. All labware and storage containers were periodically tested for endotoxin absorption and assay enhancement/inhibition.

LPS binding protein. LBP was measured using a chemiluminescent immunometric assay performed on an IMMULITE system (Diagnostic Products, Los Angeles, CA). The detectable limit of the assay was 0.2 μg/ml.

Circulating cytokines. Serum concentrations of circulating TNF-α and IL-6 (baseline) and plasma concentrations of IL-10 were assayed using a quantitative high-sensitivity (HS) sandwich enzyme immunoassay technique according to the kit manufacturer (Quantikine HS; R&D Systems, Minneapolis, MN). Assay sensitivity for TNF-α, IL-6, and IL-10 corresponded to 0.12, 0.039, and 0.5 pg/ml, respectively. Serum concentrations of IL-1 receptor antagonist (IL-1ra) and IL-6 for samples collected at 38.0–40.0°C were assayed using quantitative ELISA techniques (Quantikine; R&D Systems) with sensitivities corresponding to 22 and 0.7 pg/ml, respectively.

Antibodies and reagents. NF-κB p65 anti-human IgG_1 FITC-conjugated monoclonal antibody (MAb) and corresponding normal mouse IgG_1 isotype-matched control MAb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human MAb for cell-surface epitopes $CD16^+$ and $CD14^+$, conjugated with fluorochromes FITC and allophycocyanin (APC), were obtained from BD Biosciences. In addition, FACS-brand lysing solution, CellWASH, and CycleTest Plus DNA reagent kits were obtained from BD Biosciences. LPS (*Escherichia coli* 026:B6) and paraformaldehyde were purchased from Sigma (St. Louis, MO).

Immunofluorescence staining for intranuclear NF-κB. A whole blood flow cytometric assay for measuring NF-κB was utilized in the present study, based on an earlier technique (23). Whole blood was stained immediately using flow cytometric techniques for spontaneous intranuclear NF-κB translocation in peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophils (PMN) (see description below). In addition, at the beginning of the trial and at Exh, samples were LPS stimulated and cultured in vitro in a water bath (37°C, 30 min, 100 ng/ml) before acquisition. For each of the sampling time points, whole blood (50 μl) was incubated for 30 min with 1 ml of BD lysing solution and then processed using the BD CycleTest Plus DNA reagent kit. In brief, cells were washed once with PBS and centrifuged at 200 *g* for 5 min, and the supernatant was aspirated. The pellet was resuspended in 1.5 ml of citrate buffer and centrifuged at 300 *g* for 5 min, the supernatant was aspirated, and the process was repeated. After the second wash with citrate buffer, the supernatant was decanted before the addition of 125 μl of *solution A*

(trypsin with a spermine tetrahydrochloride detergent buffer) and 100 μl of *solution B* (trypsin inhibitor and RNase buffer in citrate stabilizing buffer with spermine tetrahydrochloride). Each solution step was incubated 10 min at room temperature. Without removal of *solutions A* and *B*, isolated nuclei were stained with either 10 μl of normal mouse IgG$_1$ (nonspecific binding) isotype control or NF-κB/p65 FITC-conjugated MAb and incubated at room temperature for 10 min in the dark. Propidium iodide (PI; 100 μl) was added to stoichiometrically bind to the clean isolated nuclei. Flow cytometric immunofluorescence acquisition was performed within 3 h of staining.

Natural killer cell staining. For phenotypic determination of natural killer (NK) cell frequency, 100 μl of sodium heparin whole blood were immediately incubated with specific fluorescent MAbs anti-CD16 FITC and anti-CD14 APC for 20 min at room temperature in the dark. Stained white blood cells were then separated from whole blood using BD lysing solution, incubated for 10 min, and centrifuged (500 *g*, 5 min), and the supernatant was aspirated. Cells were then washed (BD CellWASH) and centrifuged (500 *g*, 5 min), and the supernatant was aspirated and resuspended in 200 μl of 2% paraformaldehyde before multiparameter flow cytometric analysis. Previous work has shown that a large proportion of NK cells are CD16$^+$ and that gating on cells stained for CD3$^-$/CD16$^+$, CD16$^+$, or CD56$^+$ produces similar staining patterns in response to exercise or exertional hyperthermia compared with the conventional standard CD3$^-$/CD16$^+$/CD56$^+$ staining (78).

Flow cytometric acquisition and analyses. All samples were acquired on a FACSCalibur and analyzed using Cell Quest Pro software (BD Biosciences). For analysis of intranuclear NF-κB, double gating on a forward vs. side scatter dot plot and FL2 PI staining were used to collect 3,000 PBMC and 5,000 PMN events and to quantify intranuclear NF-κB expression in fresh whole blood and cultured samples (Fig. 2). Lymphocytes were determined using cell-surface staining characteristics gating side scatter and anti-CD14 APC, excluding monocytes and neutrophils. Two-color gating, CD14 vs.

CD16, of the lymphocyte subset was used to determine CD16$^+$ subset frequency. Total leukocyte counts were obtained from K$_2$EDTA-treated whole blood using a hematology analyzer (Coulter A^{C}T diff 2) and corrected for changes in blood volume incorporating hemoglobin and hematocrit values (19). Leukocyte subset counts (PMN, PBMC, and CD16$^+$ lymphocytes) were obtained by multiplying the corresponding population percentages obtained from FACS analysis by the total leukocyte count.

Statistical analyses. An ANOVA with one repeated factor (temperature) and one between factor (fitness) was calculated on the various immunologically and physiologically dependent measures sampled during the trial as well as a separate ANOVA comparison between baseline and Exh. In addition, a one-factor (fitness) ANOVA was used to compare physiologically dependent measures, such as BV, T$_{re}$ tolerated at Exh, TT, rate of T$_{re}$ increase, and anthropometric data. An ANOVA with two repeated factors (temperature and stimulus) and one between factor (fitness) was calculated on baseline and Exh samples stimulated in vitro. For all analyses, subject numbers (*n*) were as follows: from baseline to 38.5°C and Exh, *n* = 12 TR and 11 UT; at 39.0°C, *n* = 12 TR and 9 UT; and at 39.5°C, *n* = 11 TR. To correct for violations in the assumption of sphericity with the repeated factors, we applied the Huynh-Feldt correction to the *F* ratio. Post hoc comparisons were performed using a Newman-Keuls procedure to isolate specific group mean differences at each T$_{re}$ interval and within each group over temperature. All ANOVAs were performed with statistical software [StatSoft (2007) Statistica (data analysis software system), version 8.0, www.statsoft.com]. For all analyses, an α level of 0.05 was used.

RESULTS

Group characteristics. Anthropometric characteristics were not different between groups (Table 1). All values are means ± SE with range in parentheses. Maximum oxygen consumption expressed per kilogram of LBM [70 ± 2 (63–80) vs. 50 ± 1 (45–52) ml·kg LBM^{-1}·min^{-1}] and per unit of total mass [62 ± 2 (54–73) vs. 42 ± 1 (37–44) ml·kg^{-1}·min^{-1}] as well as body fatness [9.6 ± 1.0 (6–15) vs. 15.8 ± 1.7 (10–24)%] were significantly different between the groups (TR vs. UT, respectively). Blood and plasma volumes were also significantly elevated in TR compared with UT subjects (Table 2).

Physiological response to EHS. Our EHS model produced significant increases in HR, M$_{sk}$, and plasma volume shifts (Table 3). The absolute metabolic cost of walking at 4.5 km/h and 2% elevation was not significantly different between the groups up to 39.0°C (15.23 ± 0.1 ml·kg^{-1}·min^{-1}); however, the average relative percentage of V̇O$_{2peak}$ during the trial was significantly elevated in UT compared with TR subjects (35.9 ± 1.3 vs. 24.8 ± 0.9% V̇O$_{2peak}$, respectively). Resting HR was significantly higher in UT compared with TR subjects; however, there were no differences observed in T$_{re}$ (36.9 ± 0.1°C) at baseline between the groups. Of note, HR at exhaustion was not significantly different between groups at 160 ± 3 beats/min or 81 ± 1% of HR$_{peak}$, although still significantly lower than HR$_{peak}$ (196 ± 2 beats/min). For absolute and relative HR changes, there were significant fitness and temperature effects, greater for UT than for TR subjects. Because of lower M$_{sk}$ at baseline, change in M$_{sk}$ was significantly greater in UT compared with TR subjects for a given level of thermal strain. T$_{re}$ tolerated at Exh was higher in TR (39.7 ± 0.1°C) compared with UT subjects (39.1 ± 0.1°C), which produced significantly longer TT in TR (162.5 ± 11 min) compared with UT subjects (106 ± 10 min). However, despite the longer TT in TR subjects, the rate of T$_{re}$ increase (1.0 ± 0.05°C/h) and
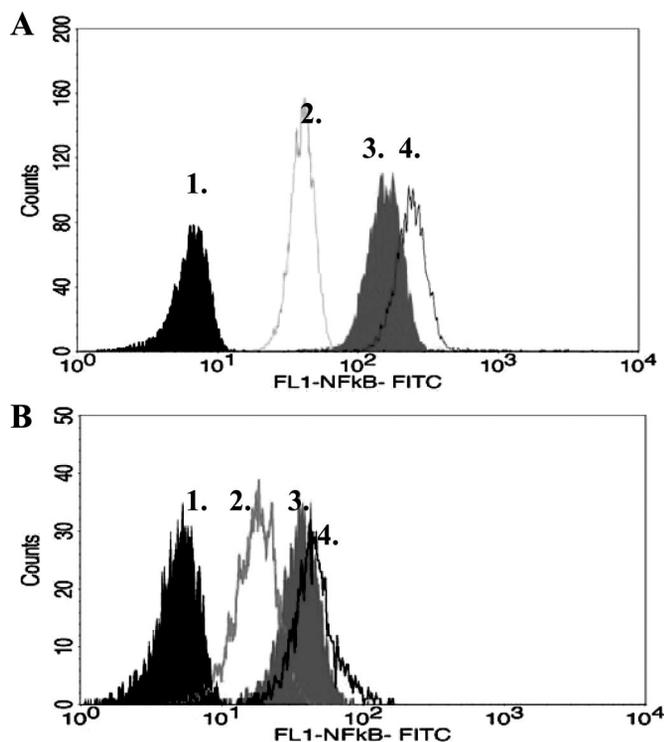

Fig. 2. Representative fluorescence histograms for NF-κB FITC staining [*1*, unstained; *2*, isotype (normal mouse IgG$_1$); *3*, NF-κB p65; *4*, NF-κB p65 in vitro LPS] for polymorphonuclear neutrophils (PMN; *A*) and peripheral blood mononuclear cells (PBMC; *B*). Data are from endurance-trained (TR) *subject 3*.

Table 1. *Anthropometric characteristics of age, height, mass, LBM, A_D, and A_D:mass for TR and UT groups*

Group	Age, yr	Height, cm	Mass, kg	LBM, kg	A_D, m^2	A_D/Mass, m$^2 \cdot$kg$^{-1} \cdot 10^{-2}$
TR	24±1 (18–31)	178±1 (170–189)	73.3±2.2 (60–89)	65.2±1.7 (55–76)	1.90±0.03 (1.78–2.11)	2.61±0.04 (2.3–2.9)
UT	23±1 (18–32)	177±2 (167–190)	78.7±2.7 (64–92)	66.1±1.6 (56–78)	1.95±0.04 (1.83–2.17)	2.50±0.04 (2.4–2.8)

Values are means ± SE (range in parentheses) for age, height, mass, lean body mass (LBM), body surface area (A_D), and surface-to-mass ratio (A_D/mass) in endurance-trained (TR) and untrained (UT) groups.

time between T_{re} sampling intervals were not significantly different between groups. Reasons for trial termination consisted of six TR subjects attaining the ethical T_{re} cutoff of 40.0°C, one UT subject attaining the HR cutoff, and the remaining 16 subjects reaching physical exhaustion. In addition, seven subjects (3 TR; 4 UT) also experienced nausea at trial termination. There was no relationship between endotoxin levels at Exh and symptoms of nausea. All osmolality values were within a normal range of 280–300 mosmol/kgH$_2$O, indicating that all subjects were euhydrated at the beginning and throughout the trial. Sweat rates were greater in TR (1.2 ± 0.09 kg/h) compared with UT subjects (0.87 ± 0.06 kg/h), and body mass was significantly decreased post-EHS, but the change in body mass was <2% (0.87 ± 0.4 vs. 1.9 ± 0.5% in UT and TR subjects, respectively), and no differences were observed between the groups. TR subjects experienced a significant plasma volume shift from baseline at 38.5°C, which remained for the duration of the trial, and were greater than values in UT subjects at Exh. In contrast, UT subjects did not experience a significant shift in plasma volume during the EHS trial (Table 3).

Total leukocytes and subset changes. EHS produced a temperature-dependent increase in the total leukocyte count and subset counts ($\times 10^9$ cells/l) (Fig. 3); however, the only detected change in leukocyte distribution was within the CD16$^+$ lymphocyte subset, with greater percentages observed in TR compared with UT subjects (Table 4).

Plasma endotoxin and LBP. Plasma endotoxin levels were not significantly different in TR compared with UT subjects at rest (trend $P = 0.1$); however, a significant group effect (UT > TR) was observed throughout the EHS trial. EHS produced significant increases in both plasma endotoxin and serum LBP levels, as depicted in Fig. 4. Resting plasma endotoxin was detectable in 11 subjects (4 TR; 7 UT), whereas at Exh, 18 of 23 subjects had a detectable increase in circulating endotoxin (9 TR, range 3.8–16.5 pg/ml; 9 UT, range 3.8–34 pg/ml). When 39.0°C was reached, a >2-fold increase in circulating plasma endotoxin concentration was observed in UT compared with TR subjects.

Intranuclear NF-κB translocation. A temperature-dependent elevation in the percentage of PBMC cells expressing NF-κB in TR and UT subjects was observed when comparing baseline to 38.0°C during EHS; however, there were no further increases in the percentage of positive cells between 38.0 and

39.0°C in UT subjects (Table 4). Increases in NF-κB in PBMC have been reported to correlate strongly with an increasing proportion of NK cells, a major source of nuclear p65 content, without intranuclear activation (69). In a whole body physical stress model, sympathetic-adrenomedullary system activation produces a redistribution of circulating lymphocytes, predominated by an increased mobilization of NK cells to the peripheral blood (78). In the present study, a significantly greater percentage of CD16$^+$ NK cells were present in TR compared with UT subjects at baseline, contributing to the greater percentage of PBMC positive for NF-κB at baseline and during EHS (Table 4). CD16$^+$ lymphocyte concentration accounted for less than one-half of the observed PBMC positive counts at baseline, indicating that other subsets, such as B and T cells and/or monocytes, might be contributing to the basal percentages. PBMC positive counts adjusted for NK cell mobilization (PBMC minus NK) produced significant increases at 38.0 and 38.5°C in UT subjects, whereas values in TR subjects did not significantly increase before 39.0°C (Fig. 5). There were no significant differences in mean fluorescence intensity (MFI) between TR and UT subjects at baseline in either PBMC or PMN. A significant main effect of temperature was observed in intranuclear NF-κB MFI in PBMC, and a significant fitness × temperature interaction was observed in PMN MFI. LPS stimulation in vitro induced significant increases in NF-κB translocation in TR and UT subjects from baseline, although at Exh, significant increases in translocation were only observed in UT subjects. Furthermore, both groups showed an attenuation of NF-κB translocation following LPS stimulation in PBMC, whereas in PMN, this was only reduced in TR subjects (Fig. 6).

Circulating cytokines. Circulating levels of TNF-α, IL-10, IL-6, and IL-1ra are depicted in Fig. 7. At rest, circulating cytokine profiles between TR and UT subjects were similar except for significantly greater levels of anti-inflammatory IL-1ra, which remained elevated in TR compared with UT subjects throughout the heat stress trial. EHS produced significant increases in proinflammatory cytokine TNF-α at a lower absolute level of thermal strain in UT (38.0°C) compared with TR subjects (38.5°C). IL-6 increased consistently throughout the heat stress trial in both groups in a temperature-dependent manner, whereas anti-inflammatory IL-1ra significantly increased at 38.0 and 39.5°C in UT and TR subjects, respectively, but concentrations in UT subjects did not reach those in

Table 2. *Absolute and relative blood volume as determined using indocyanine green dye for TR and UT individuals*

Group	n	BV, ml	BV, ml/kg	PV, liters	PV, ml/kg
TR	11	7,565±282* (6,440–9,199)	104.2±5.7* (76.5–117.5)	4,673±200* (3,621–5,482)	64.4±3.6* (45.9–89.3)
UT	10	6,675±191 (5,712–7,743)	84.0±3.1 (68.7–97.7)	4,057±100 (3,370–4,766)	51.0±1.9 (41.3–59.3)

Values are means ± SE (range in parentheses) of absolute and relative blood (BV) and plasma volume (PV) in TR and UT individuals. *$P < 0.05$, between-group difference.

Table 3. *Physiological responses for TR and UT groups during exertional heat stress*

	Baseline	38.0°C	38.5°C	39.0°C	39.5°C	Exh
HR, beats/min						
TR	60±3†	124±4*†	134±4*†	146±4*†	152±4*	156±4*
UT	75±4	140±4*	158±6*	161±4*		164±5*
Relative HR, %HR$_{peak}$						
TR	31.4±1.5†	63.3±1.7*†	68.4±1.8*†	74.5±1.8*	77.2±1.2*	79.8±5.5*
UT	38.9±1.5	71.2±1.7*	80.0±2.4*	82.2±2.1*		83.2±2.2*
M$_{sk}$, °C						
TR	34.0±0.2†	37.1±0.1*	37.4±0.1*	37.6±0.1*	37.9±0.1*	37.9±0.2*
UT	33.4±0.2	37.1±0.4*	37.4±0.4*	37.9±0.4*		37.7±0.6*
Change in PV, %						
TR	NA	−2.5±1.3	−4.2±1.4*	−6.2±2.0*	−9.1±1.4*	−7.9±1.5*†
UT	NA	−0.4±1.4	−1.8±1.2	−1.4±1.4		−2.0±1.4

Values are means ± SE. From baseline to 38.5°C and at exhaustion (Exh), $n = 12$ TR and 11 UT; at 39.0°C, $n = 12$ TR and 9 UT; and at 39.5°C, $n = 11$ TR. *$P < 0.05$, significantly different from baseline. †$P < 0.05$, between-group difference.

TR subjects. In contrast, an enhanced IL-10 concentration was observed only in TR subjects at T_{re} values above 38.5°C.

DISCUSSION

During conditions of uncompensable EHS, endurance-trained individuals have been found to tolerate $T_{re} \leq 40°C$, whereas untrained sedentary individuals succumb to EHS at much lower T_{re} values, usually around 39.0°C (77). Therefore, it was expected that in the current uncompensable EHS model, TR would tolerate a higher T_{re} at Exh. Traditionally, it has been suggested that tolerance to higher levels of T_{re} is due to an increased cardiovascular and thermoregulatory stability as produced by hypervolemia associated with training (77). The current findings suggest an additional cellular link between intranuclear NF-κB regulation and the translocation of endotoxin, which underlie the greater heat tolerance in endurance-trained individuals. This study demonstrates for the first time that increases in circulating endotoxin correspond to NF-κB translocation and inflammatory cytokine production at T_{re} values below 40.0°C. Moreover, the inflammatory cascade is accompanied by a compensatory anti-inflammatory response during uncompensable EHS. These findings support the concept of a heat illness continuum, linking the pathophysiological progression from heat stress to exertional heat exhaustion and/or exertional heat stroke (40, 79).

Redistribution of splanchnic blood and intestinal permeability. As thermoregulatory mechanisms are activated, there is a redistribution of central blood volume to the cutaneous circulation, decreasing venous return, stroke volume, and mean arterial and venous pressures (71). To maintain cardiac output with a reduced stroke volume, HR is increased to meet the demand to maintain muscle and cutaneous blood flow. Accompanying the sympathetic drive to increase HR is an equivalent sympathetic vasoconstriction of the splanchnic circulation such that corresponding relative intensities produce an equivalent reduction in visceral blood flow, independent of $\dot{V}O_{2peak}$ (70). Although a linear reduction in splanchnic blood flow is observed with increasing intensity, it does not immediately result in a compromised local oxygen demand.

A number of mechanisms are proposed for the breakdown of gastrointestinal barrier function, including the disruption of normal epithelial mucosa integrity and/or failure of epithelial physical tight junctions (20, 32). It is well documented that thermal loads above 41.5°C result in a rapid increase in intestinal epithelial permeability and onset of heat stroke (4). Heating of Caco-2 monolayers from 37 to 41°C has been found to result in increased intestinal epithelial tight junction permeability (20), and temperatures as low as 38.3°C have been found to increase transepithelial electrical conductance, resulting in paracellular permeability (51).

In addition to an increase in thermal load, adequate blood supply is critical for the maintenance of the gastrointestinal barrier (31, 72). Reduced oxygen supply impairs normal anti-inflammatory processes of the gut mucosa, resulting in increased NF-κB activation (65) and iNOS (32) and/or TNF-α release (18), which appear to be essential components for the breakdown of tight junctions and mucosa during endotoxemia (32). As such, gut ischemia is a common aspect of critical illness and has been proposed as the "motor" of multiple organ failure (12).

Reductions in visceral blood flow have been reported at HR values below 90 beats/min during light exercise (57). Yet, it appears that a significantly higher intensity is required for induction of gut ischemia-related permeability (58, 59). For instance, a significant reduction of gastric mucosal perfusion and ischemia has been reported in untrained subjects after only 10 min of exercise at 80% HR$_{peak}$ (162 beats/min) in a neutral environment (58). Comparatively, Pals et al. (59) found that 60 min of running at 80% $\dot{V}O_{2peak}$ (T_{re}, 39.6°C; HR, 180 beats/min) in trained subjects ($\dot{V}O_{2peak}$, 57 ± 2 ml·kg^{-1}·min^{-1}) was necessary to produce an increase in small intestine permeability compared with 40 and 60% intensities. Based on previous work by Rowell (70) and our current HR responses, UT subjects in the current study may have experienced a greater reduction in splanchnic blood flow compared with their TR counterparts, resulting in an earlier appearance of plasma endotoxin and TNF-α. The observed reduction in HR in TR subjects can be related to the hypervolemia associated with endurance-training, since relative to improved ventricular function, elevated blood volume increases venous return, as well as preload and subsequent venous filling pressures, and consequently, increased left ventricular end-diastolic volumes lead to an increase in stroke volume and cardiac output. This progression allows workloads to be maintained at a lower submaximal HR (28). It is interesting to note that the appearance of plasma endotoxin corresponded to the same relative HR intensity between the groups, although at significantly different levels of thermal strain: 80% in UT and 75% in TR.
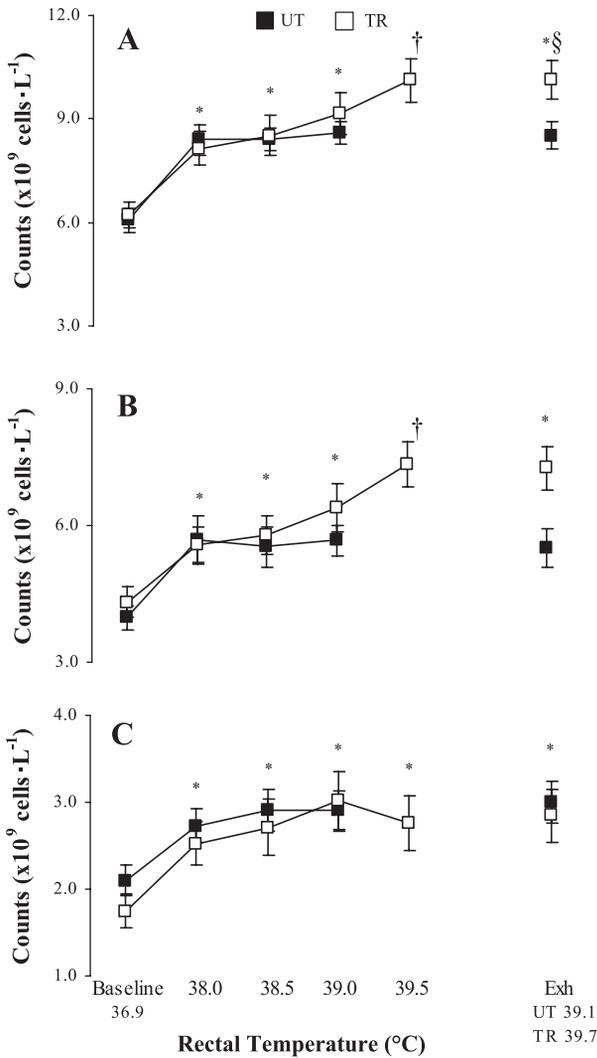
Fig. 3. Concentration ($\times 10^9$ cells/l) of total circulating leukocyte subsets during external heat stress (EHS) between TR and untrained (UT) groups for total leukocytes (*A*), PMN (*B*), and PBMC (*C*). Values are means ± SE. From baseline to 38.5°C and exhaustion (Exh), *n* = 12 TR and 11 UT subjects; at 39.0°C, *n* = 12 TR and 9 UT subjects; and at 39.5°C, *n* = 11 TR subjects. *$P < 0.05$, TR and UT are significantly different from baseline. †$P < 0.05$, TR significantly different from baseline to 38.5°C. §$P < 0.05$, between-group significance.

Fig. 4. Circulating plasma endotoxin and serum LPS binding protein (LBP) concentrations in TR and UT groups during EHS. Values are means ± SE. From baseline to 38.5°C and Exh, *n* = 12 TR and 11 UT; at 39.0°C, *n* = 12 TR and 9 UT; and at 39.5°C, *n* = 11 TR. *$P < 0.05$, UT significantly different from baseline. †$P < 0.05$, TR significantly different from baseline. ‡$P < 0.05$, UT significantly different from baseline to 38.5°C. §$P < 0.05$, between-group significance.

may have occurred at lower intensities, perhaps even below the 80% HR threshold (~160 beats/min), as suggested by previous studies (58, 59).

Overall, HR responses did not approach maximum values in either of the groups, and a HR plateau occurred before Exh despite increasing sympathetic drive (data not shown), a response that may be reflective of a protective mechanism to maintain cardiac filling pressures (36). It is unlikely that progressive fluid loss contributed to the observed differences in HR responses or reasons for trial termination between the groups, since progressive dehydration and subsequent cardiovascular drift were minimized by a regimented hydration schedule (<2% change in body weight). Previously, it was shown that a combined 1°C increase in T_{re} and 4% loss of body weight are required before significant cardiac dysfunction is observed (29).

Circulating plasma endotoxin. Quantification of endotoxin levels within the plasma and/or serum can be performed using the LAL (55). Historically, this assay has been problematic

Furthermore, the present findings are consistent with the observed appearance of endotoxin in subjects following their completion of a half-marathon (mean T_{re}, 39.6°C; HR, 172 beats/min) (53) and suggest that the onset of gut permeability
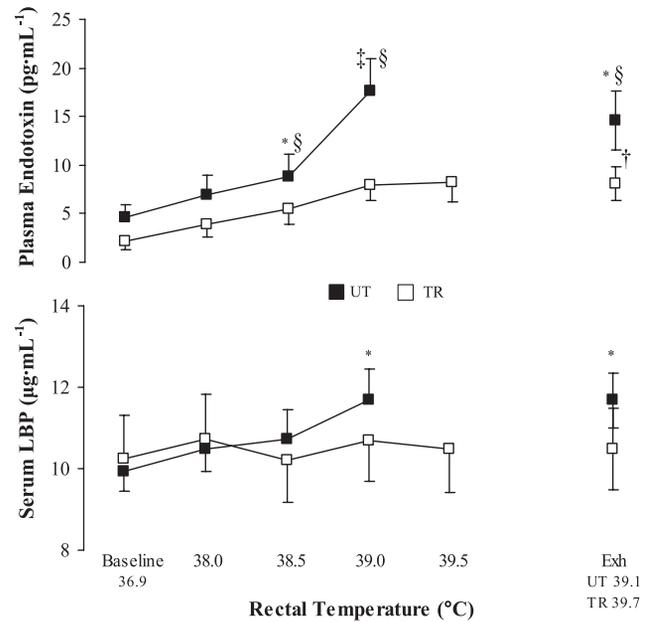
Table 4. *Percentage of PBMC positive for NF-κB and CD16$^+$ lymphocytes during exertional heat stress for TR and UT groups*

	Baseline	38.0°C	38.5°C	39.0°C	39.5°C	Exh
PBMC positive for NF-κB, %						
TR	33.9±3.8†	43.8±3.9*†	49.6±7.3*†	55.5±8.0*†	63.0±6.6*	55.1±6.1*†
UT	25.0±2.8	33.4±4.1*	36.3±4.7*	34.6±4.9*		33.0±4.6*
CD16$^+$ lymphocytes, %						
TR	19.0±2.7†	36.7±4.1*†	38.3±4.7*†	35.0±4.6*†	36.7±3.8*	35.8±4.7*†
UT	7.9±0.8	15.0±1.0*	14.0±1.1*	13.7±1.4*		13.8±1.5*

Values are means ± SE. PBMC, peripheral blood mononuclear cells. From baseline to 38.5°C and at Exh, *n* = 12 TR and 11 UT; at 39.0°C, *n* = 12 TR and 9 UT; and at 39.5°C, *n* = 11 TR. *$P < 0.05$, significantly different from baseline. †$P < 0.05$, between-group difference.
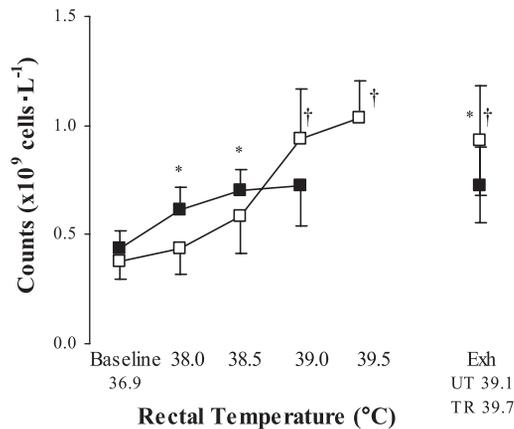
Fig. 5. Concentration ($\times 10^9$ cells/l) of PBMC positive for NF-κB corrected for CD16$^+$ natural killer counts during EHS in TR and UT groups. Values are means ± SE. From baseline to 38.5°C and Exh, n = 12 TR and 11 UT; at 39.0°C, n = 12 TR and 9 UT; and at 39.5°C, n = 11 TR. *P < 0.05, UT significantly different from baseline. †P < 0.05, TR significantly different from baseline to 38.5°C.

because of the inhibition/enhancement properties of the plasma matrix. To remove matrix interference, samples are diluted and heat treated, and a 1–3-β-D-glucan-inhibiting buffer is employed before testing is conducted. Without the incorporation of these techniques, the LAL results can be difficult to interpret. Normal background levels without β-glucan inhibition have been reported in clinical studies to occur at ∼0.05 Eu/ml (55), which, depending on the potency of the endotoxin used as a standard, corresponds to a level from 5 to 10 pg/ml in healthy controls. Nadhazi et al. (52) reported measurable levels in all of their 116 healthy donors, including a subset of lower responders who had a mean value of 0.05 Eu/ml and similar mean values of 0.06 Eu/ml that were reported in 10 student controls, ages 18–22 yr (61). Others have reported lower resting mean

levels at baseline, with a range from undetectable to 2 pg/ml (∼0.02 Eu/ml) (8, 33, 54, 56, 74). The decrease in the resting levels of endotoxin in recent studies may be attributed to the use of 1–3-β-D-glucan inhibitors. Other differences in methodology, such as heat treatment, medium for detection (plasma vs. serum) (55), and the potency of LPS used for standards, also may be contributing factors to variability in reported values. Another possible contributing factor to the variance in background values may be the nature of the subjects and individual variations in endotoxin-neutralizing capacity (88). Many of the earlier studies used age-matched normal healthy individuals as controls, whereas studies examining mild endotoxemia with strenuous exercise have employed subject cohorts consisting of marathoners/triathletes (8, 33) or ultra-endurance athletes (54). A common characteristic of the latter is a considerably higher level of aerobic fitness than would be expected in an average healthy individual. Data from current studies support the notion that training adaptations contribute to lower levels of endotoxin as have been reported in this cohort of subjects (see below).

Systemic inflammatory activation and acute phase response. As an inducible transcription factor, NF-κB is activated by a variety of agents, including reactive oxygen species (ROS) (1), cytokines (39), and endotoxin (4), many of which may be related to the pathophysiological changes associated with strenuous exercise and/or exertional hyperthermia (49, 79). Moreover, ischemic and/or thermal stress can damage the intestinal barrier, provoking the release of endogenous "danger or damage" signals from degraded tissue or necrotic cells that further initiate NF-κB activation (2). Our findings that systemic activation of NF-κB and the appearance of TNF-α in the circulation occur at a T_{re} of 38.0°C in UT subjects, preceding significant increases in plasma endotoxin, is not surprising, since endotoxemia within the portal circulation can elicit NF-κB activation and the production of inflammatory media-
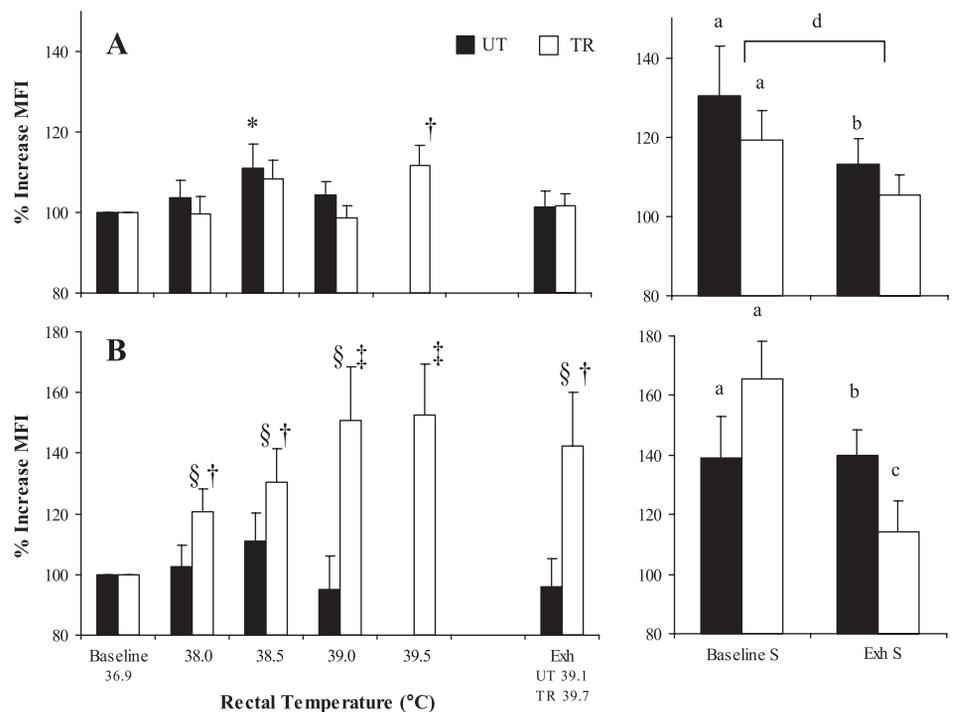
Fig. 6. Percentage change in mean fluorescence intensity (MFI) from baseline for PBMC (*A*) and PMN (*B*) positive for NF-κB in vivo and following in vitro LPS stimulation (S; 100 ng/ml, 37°C, 30 min) in TR and UT groups during EHS. Values are means ± SE. From baseline to 38.5°C and Exh, n = 12 TR and 11 UT; at 39.0°C, n = 12 TR and 9 UT; and at 39.5°C, n = 11 TR. *P < 0.05, TR and UT significantly different from baseline. †P < 0.05, TR significantly different from baseline. ‡P < 0.05, TR significantly different from baseline to 38.5°C. §P < 0.05, between-group difference. ^{a}P < 0.05, significant increase with stimulation from baseline. ^{b}P < 0.05, significant increase with stimulation from Exh. ^{c}P < 0.05, baseline S significantly different from Exh S. ^{d}P < 0.05, main effect (baseline S significantly different from Exh S).
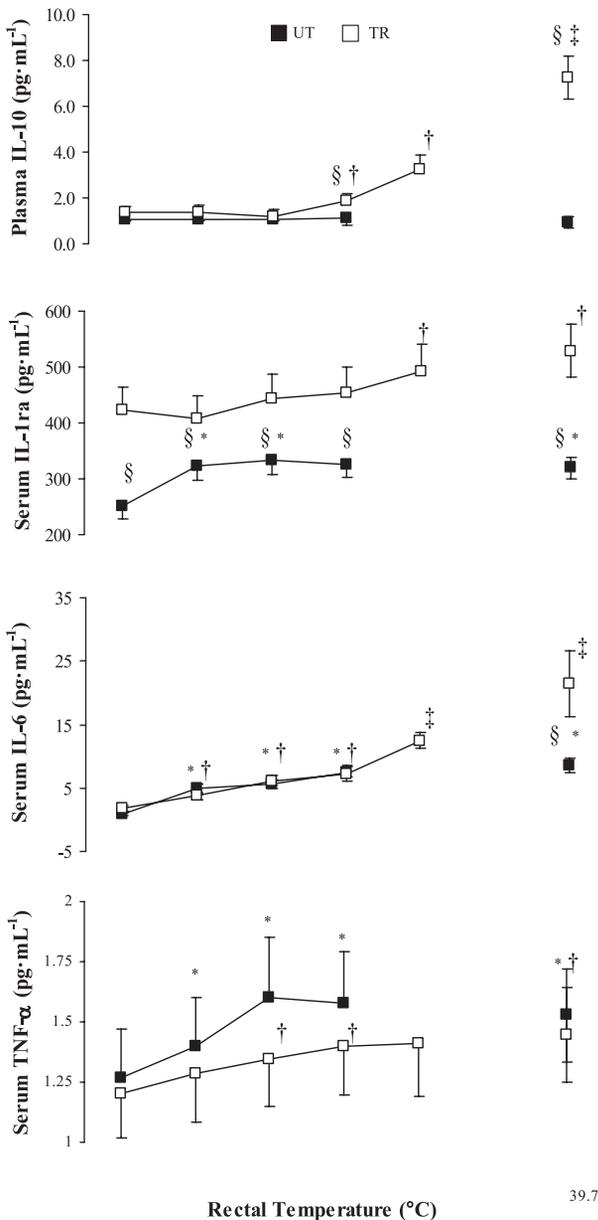
Fig. 7. Circulating plasma/serum concentrations of tumor necrosis factor (TNF)-α, IL-6, IL-1 receptor antagonist (IL-1ra), and IL-10 in TR and UT groups during EHS. Values are means ± SE. From baseline to 38.5°C and Exh, $n = 12$ TR and 11 UT; at 39.0°C, $n = 12$ TR and 9 UT; and at 39.5°C, $n = 11$ TR. *$P < 0.05$,UT significantly different from baseline. †$P < 0.05$, TR significantly different from baseline. ‡$P < 0.05$, TR significantly different from baseline to 39.5°C. §$P < 0.05$, between-group difference.

trauma (17), and in septic patients with infectious complications (91). Peak values in such disease states have been reported as high as 200 µg/ml (17). Release of LBP occurs primarily from hepatocytes and intestinal epithelial cells (35, 91) stimulated by IL-6, TNF-α, IL-1β, and/or glucocorticoids but not by endotoxin (86). Along with its ability to enhance endotoxin receptor binding, LBP at higher concentrations (50–80 µg/ml) can hinder the LBP-CD14 interaction (91), releasing bound endotoxin to lipoproteins, such as HDL (37), enhancing hepatic uptake through LDL and VLDL (87) and contributing to endotoxin-neutralizing capacity (88).

As a consequence of its critical position early in the inflammatory process, LBP has been proposed to contribute to the differences in in vitro LPS tolerance observed between trained and untrained individuals (22). Although differences in LBP concentration at baseline were not observed between our groups, LBP levels were significantly elevated in UT subjects at 39.0°C and Exh, suggesting the initiation of the acute phase response in these individuals. Despite our observed increases, LBP levels were still within the normal range (5–15 µg/ml), and it remains to be seen whether these subclinical increases enhance LPS receptor sensitivity or contribute to the reduction of in vitro LPS-induced NF-κB translocation observed post-EHS.

Circulating cytokine kinetics during SIRS are propagated by increased levels of the proinflammatory mediators TNF-α and IL-1β, followed by anti-inflammatory increases in IL-6, IL-1ra, soluble TNF receptor (sTNFR), and IL-10 in a time-dependent manner (38). By comparison, the magnitude of the cytokinemia accompanying submaximal exercise is typically much milder and does not typically include the profound increases in proinflammatory mediators associated with many clinical conditions (62, 82). Of course, there are several studies that have documented subclinical increases in proinflammatory cytokines during strenuous exercise (84) and exertional hyperthermia (60, 67, 81).

In the present study, we observed a greater increase in plasma endotoxin concentrations in UT (14.5 pg/ml) compared with TR subjects (8.08 pg/ml) at Exh, and these observed levels were comparable to those previously reported during mild endotoxemia (5–15 pg/ml) following strenuous endurance exercise (8, 33). In addition, the increases in TNF-α in the present study were comparable to those in studies employing a thermal clamping technique, which reported increases following 40–90 min of cycling at 65–70% $\dot{V}o_{2peak}$ in the heat (67, 81). Nevertheless, the observed increases in TNF-α during exertional hyperthermia are at least 100 times lower than levels seen in individuals suffering from acute heatstroke (4, 5) or following intravenous endotoxin injection (34).

Intravascular infusion of endotoxin (2 ng/kg–26 pg/ml) in healthy individuals (70 kg, 5.3-liter blood volume) has been associated with a decrease in circulating leukocytes, fever (i.e., an increase in T_{re} of 2°C within 4 h), tachycardia (HR > 100 beats/min), headache, chills, and a >100-fold increase in TNF-α (50–1,000 pg/ml) within the first hour after injection (34). The relatively low TNF-α levels observed during exertion can be partly attributed to transient TNF-α kinetics and rapid clearance from the circulation, making its detection difficult (84), especially when samples are taken at the end of the exertional period. In addition, the anti-inflammatory response associated with strenuous exercise is a regulatory adaptation

tors in both the intestinal mucosa (65) and liver (14, 26, 63) before spillover into the systemic circulation (31). In fact, both the intestinal epithelial and liver are considered important sources of inflammatory mediators during endotoxemia (13, 63). Secretion of inflammatory mediators from ischemic intestinal Caco-2 cells has even been suggested to contribute to activation of circulating PBMC and PMN priming (42).

The acute phase protein LBP is primarily responsible for transporting endotoxin to immune effector cells and can enhance sensitivity of monocytes/macrophages to LPS by more than 1,000-fold (75). Elevated plasma levels of LBP have been shown to correlate with poor outcome after thermal injury (21),

that limits pathophysiological inflammatory responses (84) and may account for the relatively low TNF-α levels observed, despite endotoxemia (53). In the present study, the subclinical increase in TNF-α (<2-fold) was accompanied by a concomitant increase in IL-6 and IL-1ra, plus a marked increase in IL-10 in TR subjects during EHS. IL-6 has been found to increase up to 100-fold (62) with strenuous exercise and can inhibit endotoxin-induced increases in TNF-α (80) as well as stimulate production of IL-1ra, IL-10 (82), and secretion of acute phase proteins in the liver (C-reactive protein and LBP) (62). Reciprocally, IL-10 can influence IL-1ra expression in LPS-challenged leukocytes (11). High levels of epinephrine produced during exercise also may blunt endotoxin-induced TNF-α release while potentiating IL-10 production (85). It is possible that increases in circulating proinflammatory mediators, such as TNF-α and IL-1β (67, 81), may be crossing a compromised blood-brain barrier during exercise in a warm environment (89), promoting physical exhaustion signaling in the brain (10) and contributing to the differences in T_{re} tolerated between our groups.

Effects of training on endotoxin-mediated cytokinemia. Knowledge of intravascular blood volume is an important factor when examining cardiovascular function and exercise performance in the heat. A major factor contributing to the increased $\dot{V}O_{2peak}$ associated with endurance training is improved cardiac function due to an increased blood volume (28, 45). As a result, hypervolemia enables endurance-trained individuals to maintain workloads at a lower submaximal HR (28). Endurance-training associated increases (20%) in relative blood volume (ml/kg) contributed to a 10% reduction in the average relative metabolic cost of exercise, HR for a given workload, and splanchnic blood flow redistribution, shifting the relationship between endotoxin-mediated cytokinemia and T_{re} to the right in these individuals (see Figs. 4, 5, and 7). This highlights an important secondary role of training-induced hypervolemia on monocytic inflammatory activation during EHS.

There is increasing evidence that translocation of small amounts of endotoxin into the circulation occurs in healthy individuals during conditions such as heat stress, which can routinely stimulate host defenses (44). Athletes training for ultra-endurance competitions, such as a marathon or triathlon, appear to have elevated levels of anti-LPS IgG before a race (3, 33). This suggests that individuals participating in regular strenuous physical activity or endurance training may develop an improved endotoxin tolerance (15, 46, 81) due to small, repeated exposures to LPS (72), resulting in a form of self-immunization (3). An important modulator of endotoxin tolerance is the repeated expression of glucocorticoids (90) and growth hormone (6), as well as anti-inflammatory cytokines and the induction of HSP in association with acute exercise (84). Although resting levels of TNF-α, IL-6, and IL-10 were not different in the present study, increased IL-1ra was observed in TR subjects, and training has been associated with increased levels of sTNFR (38). Both of these proinflammatory antagonists have been shown to enhance survival during septic conditions (62). Changes in surface expression of TLR-2 and -4 observed with training may also be important contributing factors in the improved endotoxin tolerance following habitual physical activity (22).

Another important consideration is the observation that human plasma from different individuals can exhibit more than a 100-fold range in endotoxin-neutralizing capacity (88). Endurance training has been associated with increased circulating levels of anti-LPS IgG (3, 33), IgM (7), an accentuated NK cell mobilization (68), increased levels of HDL (41), and PMN hyperreactivity, all of which may be contributing to mediate endotoxin clearance and reduce inflammatory mediators such as TNF-α or C-reactive protein in our endurance-trained subjects (83). Since the chromogenic LAL is only able to measure the potency of free endotoxin and does not account for LPS bound to various lipoproteins (LBP, HDL, VLDL, LDL) or soluble/membrane CD14, the measured levels in the present study may represent only a fraction (~30%) of the total circulating LPS content (73). Therefore, differences in baseline circulating concentrations as well as improved endotoxin tolerance may be attributed to a greater endotoxin-neutralizing capacity due to individual differences in lipoprotein profiles (41).

Induction of the stress response provides significant cytoprotection against various cellular stressors, providing maintenance of immune function (50). HSP72 accumulation has been found to complex with NF-κB/IκB, regulating inflammatory activation in the liver (14, 64), intestinal mucosa (65), and epithelium (43) during conditions of endotoxemia. A greater intracellular/circulating HSP72 induction (66) and increased HSF-1 DNA binding affinity (47) have been related to training and may contribute to the maintenance of gastrointestinal integrity at higher levels of thermal strain that was observed in our endurance-trained subjects. Likewise, impairment of transcriptional processes has been suggested in heat intolerance (49) and may hinder the ability to protect the gastrointestinal barrier against thermal and oxidative stressors in untrained, sedentary individuals.

Perspectives

Typically, the ability to tolerate higher levels of thermal load (~40.0°C) before circulatory compromise has been associated with a greater risk for the development of EHI in highly motivated endurance-trained individuals (27). Comparatively, sedentary individuals succumbing to EHS at much lower T_{re} values are less prone to EHI, as long as cessation of exertion and removal from the environment occurs. However, as indicated in the present findings, sedentary untrained individuals under significant cardiovascular and thermoregulatory strain possess compromised gastrointestinal barrier integrity and inflammatory activation at temperatures as low as 38.0°C. Since early symptoms of EHI are often misinterpreted or ignored, this may lead to a progression in EHI severity in unsuspecting individuals, driven in part by endotoxemia (40). Therefore, given the importance of the maintenance of barrier integrity and ensuing endotoxemia during EHS (4, 24, 72), the concept of endotoxin tolerance may be an important mechanism related to enhanced heat tolerance associated with endurance training. Furthermore, it is possible that transient endotoxin translocation and the subsequent induction of the stress response with repeated training bouts may be an important mediator of antioxidant and anti-inflammatory cytoprotective adaptations accompanying habitual physical activity. As our understanding of the triggers and thresholds for the initiation of the proin-

flammatory cascade and acute phase response during EHS improves, valuable insight is gained about the cellular mechanisms contributing to improved heat tolerance and the regulatory balance between proinflammatory and compensatory anti-inflammatory responses.

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