Intracellular monocyte and serum cytokine expression is modulated by exhausting exercise and cold exposure

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Rhind, Shawn G., John W. Castellani, Ingrid K. M. Brenner, Roy J. Shephard, Jiri Zamecnik, Scott J. Montain, Andrew J. Young, and Pang N. Shek. Intracellular monocyte and serum cytokine expression is modulated by exhausting exercise and cold exposure. Am J Physiol Regulatory Integrative Comp Physiol 281: R66–R75, 2001.—This study tested the hypothesis that exercise elicits monocytic cytokine expression and that prolonged cold exposure modulates such responses. Nine men (age, 24.6 ± 3.8 y; V̇O2peak, 56.8 ± 5.6 ml·kg−1·min−1) completed 7 days of exhausting exercise (aerobic, anaerobic, resistive) and under three cold, wet exposures (CW). CW trials comprised exhausting exercise and under various (aerobic, anaerobic, resistive) and under three cold, wet exposures (CW). CW trials comprised exhausting exercise and under various wet conditions. Blood monocytes are a first line of defense against invading pathogens and a major source of immunoinflammatory mediators. When activated by various noninfectious or infectious agents, such as bacteria-derived lipopolysaccharide (LPS, endotoxin), monocytes sequentially release a cascade of cytokines, including tumor necrosis factor (TNF)-α, followed by interleukin (IL)-1β, IL-6, and IL-1 receptor antagonist (IL-1ra) (2, 56). Because strenuous exercise can elicit both a pronounced monocytosis (23, 54) and systemic endotoxemia (6), it may be hypothesized that activated blood monocytes are a likely source of circulating cytokines with physical stress (7, 51). Nevertheless, studies to date have failed to directly confirm enhanced monocytic cytokine production with exercise (43) and the contribution of monocytes to exercise-induced cytokine production remains speculative (4, 7).

Although heat stress is known to accentuate exercise-associated immunomodulation, largely via augmented hormonal fluctuations (8, 45), relatively little is known regarding the physiological modulation of the human immune system by cold exposure, either at rest or during sustained exercise (50). Exposure to cold substantially augments hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) activation, producing an enhanced secretion of cortisol and catecholamines, respectively (29, 34). Cold is known to affect leukocyte mobilization (9, 30) and can suppress lymphocyte functional activities (5, 13). Further...
thermore, limited evidence suggests that cold exposure may also initiate changes in cytokine expression associated with a nonspecific acute phase reaction (9, 19, 20). Because cytokines play a key role in the bidirectional communication between neuroendocrine and immune systems (22), it has been suggested that the interplay between hormones and cytokines during thermal stress may influence immune homeostasis in response to environmental challenge (19, 29). However, the impact of sustained cold exposure on in vivo cytokine expression in humans has not been fully explored.

To better understand the regulatory role played by cytokines released during physical stress, the origin of cytokine production must be established. The present study was conducted to examine the impact of repeated bouts of strenuous, fatiguing exercise and prolonged exposures to cold, wet (CW) conditions on spontaneous and LPS-stimulated monocytic cytokine production. We hypothesized that strenuous exercise would augment cytokine expression and that exposure to cold would modify such responses. To test this, we used multiparameter flow cytometry to measure intracellular cytokine expression, which in conjunction with cell-surface marker analysis allows the frequency and functional characteristics of discrete cytokine-producing cells to be identified within heterogeneous whole blood cell populations (41). Our specific aims were 1) to study the effects of 7 consecutive days of strenuous exercise on resting and exercise-induced changes in intracellular monocytic expression of IL-1β, IL-1ra, IL-6, and TNF-α; 2) to determine if cold stress is associated with alterations in circulating and/or intracellular profiles of these cytokines; 3) to assess the relationship between circulating and intracellular cytokine expression; and 4) to examine whether changes in these mediators are associated with fluctuations in cortisol or catecholamine levels.

**METHODS**

**Subjects.** Nine healthy [peak oxygen uptake (VO$_2$ peak), 56.8 ± 5.6 (SD) ml·kg$^{-1}$·min$^{-1}$] men [age, 24.6 ± 3.8 (SD) yr; body mass, 81.3 ± 11.1 kg; body fat, 15.5 ± 5.7%; height, 1.77 ± 0.08 m] volunteered to participate in this study, which was approved by the institutional Human Use Committee. Subjects were informed of all procedures and possible risks associated with the study. After medical examination to exclude contraindications to exercise and/or cold exposure, each subject gave his written consent to participate in the experiment. Subjects were nonsmokers and were not taking prescription medications.

**Preliminary testing.** The subjects’ body composition, VO$_2$ peak, and muscular strength were assessed initially. Percent body fat was measured by dual-energy X-ray absorptiometry (Model DPX-L, Lunar, Madison, WI). VO$_2$ peak was determined by an incremental treadmill test to exhaustion and open-circuit spirometry. The one-repetition maximum of the upright row, chest press, latissimus dorsi pull-down, and biceps curl was determined.

**Experimental design.** This study took place in the summer and autumn months to minimize the possible effect of developing cold habituation. Tests were conducted over eight successive days. Subjects were tested in groups of three or four and underwent a fatiguing exercise routine for 7 days (Fig. 1). Day 1 was comprised of control measurements and CW exposure from 1330–1930 without prior exercise. On days 2–3 and 5–7, subjects performed a 4.9-km run at their personal maximal speed. Weightlifting involved one set of resistance exercises at 70% of maximum to exhaustion on rowing, benchpress, latissimus dorsi pull-down, and biceps curl movements. The mixed aerobic exercise comprised four sets of 20-min exercise bouts at ~65% VO$_2$ peak; activities, including stair-stepping, rowing ergometry, treadmill walking, upright cycling, and semirecumbent cycle ergometry. A 30-s Wingate test of anaerobic capacity concluded each day of exercise. Hiking on days 4 and 8 consisted of a 9.7-km hike over varied terrain at ~6.4 km/h while carrying a 9.1-kg backpack. Exercise was performed from 0900 to 1300 (days 2–3 and 5–7) and from 0700 to 1100 (days 4 and 8). On days 4 and 8, CW exposure was 2.5 h after the end of fatigue exercise. The CW exposure involved up to 4 h intermittent treadmill walking (six cycles of 45 min walking, 10 min of rest in simulated rain, and 5 min for transition between simulated rain and walking). Subjects attempted to complete all six cycles. During the simulated rain, subjects stood under a sprinkler delivering the equivalent of 5.4 cm rain/h while exposed to a wind velocity of 4.1 km/h, and at this stage in each cycle, they were given 250 ml of a commercial carbohydrate drink (Gatorade, Quaker Oats, Barrington, IL). Treadmill walking was performed at 5 km/h, 0% grade, with a wind velocity of 20

![Fig. 1. Experimental design from days 1 to 8.](http://ajpregu.physiology.org/Downloadedfrom/10.1152/ajpregu.00030.2017)
km/h and an average $V_{O_2}\text{peak}$ of 38.7 ± 1.4 and 39.5 ± 1.4 ml·kg$^{-1}\text{·min}^{-1}$ on days 1 and 8, respectively. The environmental chamber temperature was set at 5°C throughout the CW. Clothing consisted of a standard United States army battle dress uniform (cotton shirt, cotton-nylon jacket, cotton-nylon pants, cotton-nylon hat, socks, and leather boots, providing a thermal insulation of approximately 1.1 clo [1 clo = 0.155°C·m²/W]). During the period of simulated rain, this clothing was supplemented by a 100% nylon rain hat and nylon gaiters. The CW was terminated if the subject’s core temperature dropped below 35°C, or if the subject requested to stop.

**Temperature measurements.** A thermistor inserted 10 cm past the anal sphincter and an automated data-acquisition system measured the rectal temperature at 1-s intervals.

**Blood sampling schedule.** Peripheral blood samples, totaling 21 ml per determination, were drawn through an 18-gauge 5-cm intravenous catheter inserted into the left antecubital vein while the subjects sat quietly at rest (0700) 30 min after fatiguing exercise (~1130) and 30 min after CW (~1600) on days 1 and 8 (Fig. 1). We maintained catheter patency by flushing the device with heparinized saline (10 units/ml). Blood samples were collected into plastic syringes and transferred immediately to prechilled glass tubes containing specific anticoagulant for plasma or nonadditive tubes for serum.

**Circulating cytokine assays.** Serum concentrations of IL-1, IL-1ra, IL-6, and TNF-α were measured according to the manufacturer's (Quantikine, R&D Systems, Minneapolis, MN) instructions, using solid-phase sandwich ELISA kits with sensitivities of 0.3, 22, 0.7, and 0.2 pg/ml, respectively. Optical density, with wavelength correction, was read on an automated microplate photometer (EL340, BIO-TEK Instruments, Winooski, VT).

**Antibodies and reagents.** Mouse anti-human monoclonal antibodies (mAbs), directly conjugated with the fluorochromes fluorescein isothiocyanate (FITC) and phycoerythrin (PE) against the cell-surface epitope CD14-FITC and Fast Immune anti-human-cytokine mAbs specific for IL-1-PE, IL-1ra-PE, IL-6-PE, TNF-α-PE, along with their respective isotype-matched (IgG1 and IgG2a) control mAbs, were obtained from Becton Dickinson Biosciences (San Jose, CA). FACS-brand lysing solution, permeabilization solution, and CellWASH (optimized PBS containing 0.1% sodium azide) were also obtained from Becton Dickinson Biosciences. Paraformaldehyde, LPS, *Escherichia coli* 055:B5, and brefeldin A (BFA) were purchased from Sigma (St. Louis, MO).

**Cell preparation and culture.** Heparin sodium-anticoagulated whole blood was immediately treated with 60 µl of BFA (at a final concentration of 10 µg/ml) to promote the accumulation of de novo synthesized cytokines within the Golgi apparatus of the synthesizing cell. Next, BFA-treated blood was aliquoted into 12 × 75-mm poly styrene Falcon tubes (Becton Dickinson Biosciences). One milliliter of blood was used for determination of unstimulated or spontaneous intracellular cytokine expression. Elicited cytokine expression was determined by stimulating a second 1-ml aliquot with a predetermined, optimal concentration (1 µg/ml) of LPS dissolved in sterile pyrogen-free PBS, for 4 h in a 5% CO₂ humidified atmosphere at 37°C.

**Two-color staining for intracellular cytokine production.** For phenotypic determination of CD14<sup>+</sup> monocyte frequency, 100 µl aliquots of unstimulated and in vitro LPS-stimulated whole blood were incubated with saturating concentrations of anti-CD14-FITC surface stain for 15 min at room temperature in the dark. Immediately after incubation, cells were treated for 10 min with 2 ml of 1× FACS lysing solution. After centrifugation (5 min, 500 × g), cell membranes were treated for 10 min with 2 ml of 1× FACS permeabilizing solution and incubated for 30 min with anti-cytokine-PE antibodies against IL-1, IL-1ra, IL-6, and TNF-α. After being incubated and washed with 2 ml of CellWASH, the cell pellets were resuspended in 300 µl of 2% paraformaldehyde before analysis on a flow cytometer.

**Flow cytometric acquisition and analysis.** Stained cell suspensions were acquired on a dual-laser EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL) calibrated for two-color analysis. An electronic acquisition gate was set on CD14<sup>+</sup> cells according to FITC emission and 90° side scatter light scattering. Typically, ≥5,000 CD14<sup>+</sup> monocyte-gated events were acquired for analysis of the frequency of intracellular cytokine staining. Analysis gates and quadrant markers were set to define positive and negative populations for cytokine production, according to the staining of isotype-matched negative controls. Results were expressed as the percentage of cytokine-positive monocytes in unstimulated and LPS-stimulated cultures. Absolute monocyte counts were obtained by multiplying the corresponding percentages of CD14<sup>+</sup> cells derived from FACS analysis with the total leukocyte counts derived from a hematology analyzer (Coulter Electronics). All postexercise values were adjusted for changes in blood volume according to the equations of Dill and Costill, using hemoglobin and hematocrit values (16).

**Hormonal analyses.** Specimens for catecholamine (epinephrine (Epi) and norepinephrine (NE)) determination were stored briefly on ice in 4.5-ml tubes containing EDTA and reduced glutathione (Amersham, Arlington Heights, IL). The supernatant plasma was separated in a refrigerated centrifuge for 15 min (4°C; 3,000 × g) and frozen at −80°C until assay. Unbound plasma catecholamine concentrations were quantitated by gas chromatography-mass spectrometry. We measured total serum concentrations of cortisol, using an IMMULITE chemiluminescent immunoassay system (Diagnostic Products, Los Angeles, CA). Postexercise hormone levels were corrected for changes in plasma volume.

**Statistical analyses.** Two-way repeated-measures (trial × time) ANOVA were used to evaluate differences. When significant $F$ ratios were calculated, Neuman-Keuls post hoc analyses were made to isolate differences among treatment means. Separate stepwise multiple regression analyses were completed for cell counts and circulating and intracellular cytokines, and we compared each of the hormonal responses (independent variables) to each of the cellular subsets or cytokines (dependent variables). Data are presented as means ± SE, and the level of statistical significances was set at $P < 0.05$ for all analyses.

**RESULTS**

**Thermal response.** Cold exposure times averaged 5.2 ± 1.1 and 4.8 ± 1.3 h on days 1 and 8, respectively. Before entering the cold chamber on these days, the subjects had mean rectal temperatures ($T_{re}$) of 37.1 ± 0.25 and 37.2 ± 0.25°C, respectively. $T_{re}$ declined significantly during cold exposure on each day (day 1, 36.89 ± 0.86; day 8, 36.9 ± 0.78°C). $T_{re}$ was significantly higher in the second and third hours of cold exposure on day 8 compared with day 1, with no difference between trials for the last 3 h of exposure. Details of subject attrition, cold tolerance, and other thermoregulatory responses have been reported elsewhere (11).
Total leukocyte and monocyte counts. Resting (0700) leukocyte (4.96 ± 1.65 × 10⁹/l) and monocyte (0.39 ± 0.13 × 10⁹/l) concentrations on day 1 were within reported normal ranges (Table 1), and values remained unchanged on day 8, after 1 wk of exhausting exercise. Total leukocyte [F(2,16) = 34.8, P = 0.0001] and monocyte [F(2,16) = 10, P = 0.002] counts showed significant main effects for time. Significant trial × time interaction effects were also detected for both cell subsets. Post hoc analyses showed elevated leukocyte (6.82 ± 1.28 × 10⁹/l) and monocyte (0.54 ± 0.11 × 10⁹/l) counts postexercise on day 8 (1130) compared with day 1 and with resting values (0700) within trials. After the 6-h CW (2000), leukocyte (≥12.4 ± 3.97 × 10⁹/l) and monocyte (≥0.72 ± 0.33 × 10⁹/l) counts were significantly elevated on both days 1 and 8, with no differences between trials.

Circulating cytokine concentrations. Spontaneous serum cytokine concentrations were low in resting subjects on day 1 (Fig. 2). TNF-α levels were below detection limits at 0700 on day 1, and IL-1β remained undetectable throughout the study. There were no significant differences between resting cytokine values on days 1 and 8. Significant main effects of time were found for IL-1ra [F(2,14) = 31.7, P = 0.0001], IL-6 [F(2,14) = 32.3, P = 0.0001] and TNF-α [F(2,14) = 17.6, P = 0.001]. Circulating cytokine levels did not change when subjects were at rest at 1130 on day 1. Exercise on day 8 (1130) led to significantly increased concentrations of IL-1ra (804 ± 250 pg/ml), IL-6 (9.4 ± 2.1 pg/ml), and TNF-α (3.2 ± 0.7 pg/ml). Similarly, the CW on day 1 increased concentrations of all three cytokines (all P < 0.0001). On day 8, the CW further augmented IL-1ra (1238 ± 414 pg/ml) and IL-6 (13.8 ± 6.2 pg/ml) concentrations but caused TNF-α levels to return to baseline. Significant interaction effects were obtained for IL-1ra [F(4,28) = 3.7, P < 0.05], IL-6 [F(4,28) = 9.5, P < 0.05], and TNF-α [F(4,28) = 39.8, P = 0.001], with intertrial differences isolated to the 1130 and 2000 time points.

Intracellular cytokine expression. Constitutive and induced intracellular IL-1β, IL-1ra, IL-6, and TNF-α production by BFA-treated whole blood samples are shown in Fig. 3. The percentage of cytokine-positive monocytes spontaneously exhibiting expression of all four cytokines by unstimulated CD14+ monocytes was low in resting (0700) subjects on day 1. Positive staining for IL-1β (2.8 ± 1.4%) and IL-1ra (4.7 ± 1.5%) was greater than for IL-6 (2.3 ± 0.8%) and TNF-α (2.1 ± 1.3%). Significant main effects across time were observed for IL-1β [F(2,12) = 37.8, P = 0.0001], IL-1ra [F(2,10) = 17.6, P = 0.001], IL-6 [F(2,8) = 11, P = 0.005], and TNF-α [F(2,8) = 8.5, P = 0.01]. Relative to unstimulated samples, 4 h of LPS stimulation (1 μg/
ml) increased the percentage of cytokine-producing monocytes several thousandfold at all time points \( (P < 0.0001) \). Neither stimulated (LPS+) nor unstimulated (LPS−) cultures showed any differences in cytokine expression at 1130 on day 1 relative to initial values (0700). After the CW (2000) on day 1, the percentages of IL-1ra (8.5 ± 2.8%), IL-6 (7.6 ± 1.8%), and TNF-α (3.3 ± 0.5%) among unstimulated CD14+ monocytes were significantly increased, but there was no change in unstimulated intracellular IL-1β expression.

After 1 wk of exhausting exercise (day 8), resting IL-1β levels (6.5 ± 1.3%) were significantly elevated relative to day 1, but resting expression of the other three cytokines was unchanged. Acute exercise increased the expression of all four cytokines in unstimulated samples (Fig. 3). The CW further enhanced IL-1ra expression (12 ± 3.7%); in contrast, cold exposure caused a significant reduction in the expression of unstimulated IL-1β (1.1 ± 0.5%), and TNF-α expression dropped below baseline by the end of the CW on day 8. Both exercise and the CW significantly increased the ex vivo capacity of LPS-stimulated whole blood cultures to form IL-1β and IL-6, but exercise in the cold suppressed TNF-α (67.8 ± 6.6%) formation on day 8. The intracellular expression of IL-1ra was consistently higher (≥90%) than that of other cytokines in response to LPS challenge but was unaltered by either acute exercise or the CW. In contrast to unstimulated IL-6 expression, LPS-stimulated production of IL-6 was increased slightly (70.3 ± 7.9%) at rest on day 8.

Circulating hormone concentrations. Resting NE and Epi concentrations were comparable between trials (Fig. 4). However, there were significant main effects for NE \( [F(2,16) = 49.8, P = 0.0001] \), Epi \( [F(2,16) = 38.9, P = 0.0001] \), and cortisol \( [F(2,16) = 11.4, P = 0.001] \) over time. Post hoc analysis traced the differences in catecholamines to significant elevations of both NE and Epi after the CW on both days 1 and 8. Neither NE nor Epi showed significant trial × time interaction effects. However, a significant interaction effect \( [F(4,24) = 4.2, P = 0.04] \) was found for circulating cortisol. Peak cortisol values occurred in the early morning (0700) on both days 1 and 8, with values dropping significantly by 1130. Compared with resting preexposure values on day 1, the CW conditions stimulated cortisol secretion significantly on both days 1 and 8. No intertrial differences were found between 1130 and 2000 sample times.
elevated resting (0700) cortisol levels were observed on day 8 compared with day 1.

**Regression analyses.** Relationships among serum hormone concentrations, cell counts, and intracellular and circulating cytokines derived by stepwise multiple regression are presented in Table 2. The proportion of total variance ($R^2$) attributed to changes in concentrations of Epi and NE ranged from 38–49% for circulating cell counts and 34–40% for intracellular cytokine expression. Intracellular IL-6 was the only cytokine whose expression was significantly ($R^2 = 0.342, P = 0.02$) related to serum cortisol levels. Circulating IL-6 concentration was also positively associated ($R^2 = 0.261, P = 0.04$) with NE, but changes in IL-1ra and TNF-α levels were unrelated to any hormone concentrations. Simple linear regression analyses identified positive correlations between intracellular expression and circulating concentrations of IL-1ra ($r = 0.668, P = 0.002, 7$ degrees of freedom (df)) and IL-6 ($r = 0.504, P = 0.03, 7$ df) postexercise. No relationship was observed between intracellular and circulating TNF-α levels, but increases in intracellular IL-6 after exercise showed a strong positive association with intracellular IL-1ra expression ($r = 0.759, P = 0.0003, 7$ df).

**DISCUSSION**

This report demonstrates that physical exercise triggers human peripheral blood monocytes to express enhanced levels of IL-1β, IL-1ra, IL-6, and TNF-α and that upregulated intracellular expression of inflammation-associated cytokines mainly corresponds with their increased serum concentrations. We have also shown that prolonged exposure to cold, wet environmental conditions differentially modulates cytokine expression. Furthermore, our findings provide evidence to suggest that changes in sympathoadrenal activation are linked to exertional and cold-induced modification of these cytokine production profiles.

Although numerous sources of cytokines have been identified in vitro, relatively few studies involving exercise have attempted to identify the origin of cytokines in vivo (4, 43). Previous research has been limited mainly to bulk ELISA measurements of IL-6, TNF-α, IL-1β, and more recently IL-1ra in the circula-

**Table 2. Relationship between intracellular and circulating cytokines to changes in serum hormone concentrations by stepwise multiple regression**

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<tr>
<th>Independent Variables</th>
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Probabilities are expressed as $P$ values for individual regression coefficients. Intracellular cytokine values are from unstimulated (without lipopolysaccharide) whole blood samples only. Circulating IL-1β levels were below the detection threshold of the assay. IL, interleukin; ra, receptor antagonist; TNF, tumor necrosis factor; $R^2$, coefficient of determination; Epi, epinephrine; NE, norepinephrine; NS, not significant ($P > 0.05$). *Negative association between variables.
localized cytokine production, upregulated IL-1 inhibitors (13, 20, 47). Supporting the importance of such catabolism and/or competition with circulating IL-1 antago-
sic monocytes expressing each of the four cytokines revealed significant postexercise increases in the frequency of monocytes expressing each of the four cytokines assayed. The greatest exercise-associated increases were detected for intracellular expression of IL-6 and IL-1ra, followed by IL-1β and TNF-α. These findings provide the first direct evidence that blood monocytes can be a source of circulating inflammatory cytokine production with exercise. In accordance with our results, previous human studies (28, 49) have indicated that among circulating immunocytes, unstimu-
lated CD14+ monocytes are the primary source of these inflammatory-associated cytokines in vivo. Such conclusions are strengthened by the close correlations observed between spontaneous intracellular expression of IL-6 and IL-1ra and between intracellular expression and circulating concentrations of these cytokines. Furthermore, our ex vivo findings that exercise increased circulating IL-6 and TNF-α levels by 273 and 83%, respectively, are consistent with the literature showing proportionally greater elevations of IL-6 than TNF-α (25, 31, 39). Similarly, the marked elevation (113%) of circulating IL-1ra observed postexercise is in agreement with prior exercise studies (18, 37, 39). The fact that we observed significant intracellular expression of IL-1β in the absence of a circulating rise in this cytokine implies that, although local monocytic IL-1β production is enhanced shortly after exercise, its systemic accumulation may be prevented by rapid catabolism and/or competition with circulating IL-1 antagonists (13, 20, 47). Supporting the importance of such localized cytokine production, upregulated IL-1β and IL-6 expression has been found in isolated skeletal muscle preparations after exercise (10, 40, 53). However, it remains unclear whether infiltrating monocytes or active myofibrils themselves are the primary source of these cytokines within muscle (27, 51).

Our study demonstrates that fatiguing exercise significantly enhances LPS-stimulated intracellular expression of IL-6 and TNF-α-positive monocytes. This observation supports earlier in vitro data showing that exercise does not significantly impair cytokine synthesis in response to LPS challenge (4, 15, 38, 58). In contrast, LPS-stimulated production of IL-1ra was unchanged by our experimental treatment, which probably reflects the excess capacity of monocytes to produce IL-1ra even under basal conditions (17).

The modest elevation in CD14+ monocytes (~50%) that was detected postexercise is comparable to observations from other studies measuring cellular redistribution with similar exercise designs (7, 45). Typically, monocytes are deployed rapidly from the marginal pool into the circulation during the first minutes of strenuous exercise (23), but their numbers drop quickly on cessation of activity (7). Under the current protocol, prolonged cold exposure substantially magnified the extent of monocytosis (~100%), regardless of whether or not the subjects had performed prior fatiguing exercise. Such cold-enhanced recruitment of monocytes has been previously documented in humans (9, 30) and is presumably mediated by pronounced SNS activation accompanying prolonged cold stress. This activation may influence cell mobilization through indirect adjustments in hemodynamics or via direct receptor-mediated alterations in cellular adhesive properties, thereby affecting cell mobilization (19). In sum, these findings strengthen the hypothesis that strenuous exercise selectively mobilizes those monocytes with an enhanced functional capacity (23, 54).

Another important finding of this investigation is that when fatiguing exercise preceded cold exposure, the spontaneous intracellular expression of IL-1β and TNF-α was substantially reduced. Furthermore, in vitro intracellular monocytic expression of TNF-α and its serum levels were also markedly suppressed in response to LPS challenge after cold exposure. Such results are consistent with results of a recent study (20) demonstrating a blunted secretion of IL-1β and TNF-α by monocytes cultured at 32°C compared with levels at 37°C. By comparison, we found unstimulated expression of IL-1ra and IL-6 to be enhanced after cold exposure. This agrees with our previously reported finding that short duration, moderate cold-air exposure (2 h, 5°C) in a climactic chamber elicits significant plasma elevations of IL-6 in resting subjects (9), consistent with a report of higher IL-6 levels in healthy volunteers after they swam in ice-cold water (19). Others have found that short-term exposure to cold air (1 h, 11°C) or cold water (1 h, 14°C) has no effect on systemic IL-1β or IL-6 release (30, 35), although it does augment circulating TNF-α production (30). On the other hand, studies of perioperative and accidental hypothermia have demonstrated that surface cooling to a core temperature of 34–30°C suppresses systemic IL-1β and IL-6 production after surgery or traumatic injury, respectively (1, 5). By contrast, a recent case study of two extreme hypothermia (core temperature ≤28°C) victims reported greatly augmented circulating IL-6 levels on admission to the hospital (1). Thus it appears that different mechanisms of cytokine induc-
The mechanism underlying the abovementioned differences in cytokine generation is not clear, but it can be argued that cold-associated modulation of cytokine production may be related to induction of systemic endotoxemia, provoked by alterations in central hemodynamics and stress hormone release associated with enhanced thermoregulatory demands. In support of this notion is the observation that moderate cold exposure leads to a sharp reduction in splanchnic blood flow and ischemia (24) that promotes translocation of LPS into the systemic circulation (26). Also, on rewarming, the potential for even greater endotoxemia exists due to transient splanchnic reperfusion (24). Moreover, cold exposure may further exacerbate such effects via enhanced sympathoadrenal activation (48) and/or by directly augmenting the biological activity of LPS (32). Based on this evidence, we hypothesize that under the current experimental conditions, cold exposure is likely to have facilitated endogenous cytokine release by LPS-activated circulating monocytes.

Although the present data support the view that circulating monocytes can be a source of cytokines with exercise, they do not per se exclude other immune and/or accessory cells as potential contributors to overall cytokine production. In fact, our findings contradict previous in vitro studies that failed to demonstrate exercise-induced changes in the expression of monocyte mRNA and/or protein levels of IL-1β, IL-6, and TNF-α (40, 47, 55). Such findings have led some investigators (4, 37, 43) to speculate that noncirculating cells, including vascular endothelium, hepatocytes, and/or fibroblasts, may be chiefly responsible for the enhanced secretion of these cytokines with exercise. Inconsistencies concerning the capacity of exercise to elicit mononuclear cytokine production may be, at least partially, attributable to methodological differences between studies. For example, compared with previous experiments, the extremely fatiguing exercise regimen used in the current model may have provided a stronger stimulus for monocyte activation and thereby greater cytokine synthesis. In addition, the sensitivity of intracellular flow cytometric techniques for enumerating cytokine-producing cells is superior to earlier methodologies (33). Finally, our use of whole blood, rather than isolated mononuclear cells, more reliably simulates the complex in vivo cellular and humoral milieu (14).

Data from the present study showing significant correlations between circulating hormones and cytokine expression appear to support an important role for reciprocal interactions between neuroendocrine and immune systems (22) in the maintenance of homeostatic balance between pro- and antiinflammatory cytokine responses (17). For instance, IL-6 is induced along with other cytokines during the inflammatory cascade but does not mediate proinflammatory symptoms (46). Instead, IL-6 activates the HPA axis and induces the upregulation of cortisol and IL-1ra that in turn suppresses the synthesis of mononuclear IL-1β, TNF-α, and IL-6 (17), thereby controlling the extent of local and systemic inflammatory responses. In this study, the positive correlation observed between circulating IL-6 levels and NE also agrees with previous findings that exercise- and cold-induced catecholamine secretion is closely related to systemic IL-6 release (9, 42). Likewise, the positive association of NE and Epi concentrations with mononuclear IL-6 and IL-1ra expression, but negative association with IL-1β and TNF-α, suggests that cytokine production may be differentially regulated by circulating catecholamines during exercise and cold exposure.

In conclusion, flow cytometric detection of intracellular cytokines has proven to be a useful tool to study cytokine expression at the single cell level, thus improving our understanding of the cellular sources of cytokines during physical stress. The present findings demonstrate that blood monocytes are a source of IL-1β, IL-1ra, IL-6, and TNF-α production after acute strenuous exercise. In addition, prolonged cold exposure was shown to differentially modulate cytokine production, upregulating the expression of IL-6 and IL-1ra but downregulating that of IL-1β and TNF-α. Secretion of sympathoadrenal hormones was significantly associated with the changes in both circulating and intracellular cytokine profiles. These findings suggest that multiple interactions between cytokines and neuroendocrine hormones are likely involved in the physiological response to exertional fatigue and cold and may serve to limit the severity of the host inflammatory response. Because the application of intracellular cytokine flow cytometry is a novel approach to studying exercise and thermally mediated cytokine modulation, future research is warranted to investigate the possible contribution of additional immune cells and/or immune accessory cells to such responses.

Perspectives

The molecular signaling pathways involved in exercise- and/or thermal stress-induced cytokine alterations remain largely unknown. The current findings are consistent with studies indicating that adrenergic/noradrenergic mechanisms are intimately involved in the regulation of cytokine production under various forms of physical stress (3). Monocytes express both α- and β-adrenergic receptors, and binding of Epi/NE can activate or inhibit different signal transduction pathways (44). The stimulation of β2-adrenoceptors during stress attenuates excessive synthesis of proinflammatory cytokines (IL-1β, TNF-α) and elevates antiinflammatory cytokines (IL-6, IL-1ra, IL-10) via increased cAMP (44, 59). However, activation of α7-adrenoceptors is associated with reduced cAMP levels and enhances proinflammatory cytokine synthesis (57). In this context, the current observations showing that cold exposure elicited differential changes in IL-1β and TNF-α suggest that α-adrenergic mechanisms were able to prevail after cold stress on day 1. In contrast, cold exposure on day 8 decreased mononuclear TNF-α and IL-1β but stimulated IL-1ra expression, indicating...
that β-adrenergic mechanisms may have predominated when cold stress was preceded by fatigue exercise. Alternatively, it is conceivable that exhausting exercise for 7 days may have altered monocyte adrenoceptor density (3), thereby reducing the capacity for excessive synthesis of proinflammatory cytokines but enhancing antiinflammatory cytokines after cold exposure. Conclusive evidence for such cold-evoked, SNS-associated modulation of cytokine expression must await future studies that interdict specific steps in the signaling pathways leading to cytokine induction.

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