Acute stressor exposure facilitates innate immunity more in physically active than in sedentary rats

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Acute stressor exposure facilitates innate immunity more in physically active than in sedentary rats. Am J Physiol Regulatory Integrative Comp Physiol 282: R1680–R1686, 2002; 10.1152/ajpregu.00661.2001.—Most previous stress-immune research focused on the immunosuppressive effects of stress on acquired immunity. More recently, it has become clear that acute stressor exposure can potentiate innate, as well as suppress acquired, immunity. For example, acute stress improves recovery from bacterial inflammation, a classic in vivo measure of innate immunity. The previous work was done in sedentary organisms. Physical activity status can modulate the impact of stress on immune function. The following studies tested the hypothesis that the effect of stress on inflammation after subcutaneous challenge with bacteria (Escherichia coli) is facilitated by physical activity. The results were that sedentary, stressed rats resolved their inflammation 1–2 days faster and have increased circulating neutrophils compared with their nonstressed, sedentary counterparts. In contrast, physically active, stressed rats resolve their inflammation 3–4 days faster and have increased circulating and inflammatory site neutrophils compared with their nonstressed counterparts. Importantly, the beneficial impact of stress on inflammation recovery and neutrophil migration was greater in the physically active, than sedentary, stressed rats. Thus physical activity status facilitates the positive effect of acute stress on innate immunity.

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be easily monitored. Second, nonreplicating bacteria do not induce behavioral confounds associated with the generation of sickness or death (7). Third, inflammation occurs quickly (within hours of injection) and is generated and resolved by primarily neutrophils and macrophages, the two primary cells of innate immunity (2). Finally, the injection of streptomycin-treated bacteria triggers an inflammatory response without involving a nonreplicating signal; thus any potential direct effect of hormones released in response to stressor exposure on bacteria growth cannot confound the interpretation of the results (25).

In addition, several potential mechanisms that participate in the bacterial inflammatory response were examined. Specifically, changes in neutrophil numbers (in blood and at the inflammatory site), dermis vascularization (capillary density at the inflammatory site), edema at the inflammatory site, and blood complement activity (C3-mediated lysis) were measured after stressor exposure in both sedentary and physically active rats. Increases in any of these factors due to stress and/or physical activity could facilitate recovery from bacterial infection.

The following studies, therefore, will test the hypothesis that regular, moderate, and voluntary physical activity will modulate the impact of stress on immunity. Specifically, we hypothesize that the physically active organisms will benefit most from the stimulatory effect of stress on innate immunity.

METHODS

Subjects. Adult male Harlan Sprague-Dawley specific pathogen-free rats (275–300 g) were used in all experiments (5–10 animals/experimental group). All subjects were maintained on a 12:12-h light-dark cycle (lights on 0600 to 1800) and housed in a specific-pathogen-free barrier facility. Subjects were allowed to acclimate to the colony for 14 days before experimentation began. They were handled briefly once daily for 3 days just before the start of the experiment. Colony room temperature was maintained at 22–23 °C. Food and water were available ad libitum. Care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

IS procedure. Animals either remained in their home cages or were placed in Plexiglas restraining tubes (23.4-cm long and 7.0-cm diameter) and exposed to 100 5-s 1.6-mA IS, with an average intertrial interval of 60 s, through electrodes attached to the tail. All animals were shocked between 0800 and 1000.

Freewheel running. Rats were housed in standard Nalgene Plexiglas cages (18.7 × 10.5 × 6.1) with a stainless steel open running wheel attached (19.5 × 10.4 × 14.2). Physically active rats had a mobile running wheel and ran for 6–8 wk before stressor exposure. Sedentary controls were housed in the same environment except that the running wheel was locked and remained immobile. The caging environment meets National Institutes of Health (NIH) floor space standards for a single rodent. Total daily running distances were monitored by computer with the VitalView Automated Data Acquisition System (Sunny River, OR). Rats were weighed weekly.

Bacterial culture. E. coli (ATCC 15746) were purchased from American Type Culture Collection (Bethesda, MD). Vial contents were rehydrated and grown overnight to maximal densities in 30 ml of brain-heart infusion (BHI; DIFCO Laboratories, Detroit, MI) or nutrient broth cultures, respectively (37 °C; 95% air-5% CO2). Cultures were then aliquoted into 1.0 ml nutrient broth supplemented with 10% glycerol and frozen at −70 °C. These vials constituted the stock cultures. Two days before the study, stock cultures were thawed and cultured overnight in 30 ml of BHI or nutrient broth. The next day, new cultures were started by adding 100 μl of the overnight culture to 30-ml vials of BHI and cultured at 37 °C, 95% air-5% CO2. When cultures reached midlog growth (determined by reading absorbances at 595 nm), 1 ml of sterile filtered streptomycin (150 mg in 1 ml) was added to streptomycin-treated cultures, and 1 ml of sterile PBS (Life Technologies, Grand Island, NY) was added to the control culture. A final reading was performed 4 h later to compare streptomycin-treated with control cultures. The number of bacteria in cultures was then extrapolated from previously determined growth curves. Verification that the bacteria was killed by the streptomycin was performed by plating the cultures on Tryptic Soy Agar (DIFCO Laboratories) plates. Consistent with our previous work (12), no detectable bacterial growth was noted after 2 days in culture (data not shown). Cultures were then centrifuged for 15 min at 3,000 rpm, supernatants were discarded, and bacteria was resuspended in sterile PBS. Dead bacteria was stored at 4 °C until the next day at which time 2.5 × 106 CFU were injected subcutaneously into the rats.

Bacterial challenge and in vivo inflammation assessment. One day before injection, an area on the rat’s dorsal surface measuring ~4 × 4 cm was shaved so that inflammation could be easily quantified. On the day of the study, subcutaneous injections were given in a volume of 500 μl just posterior to the shoulder blades. All injections were administered between 1000 and 1200 immediately after stressor termination. Rats were then returned to their home cages. The diameter of inflammation (to the nearest mm) was always measured between 0800 and 1100 using a standard caliper. Bacterial inflammation grade assessments were also made daily. The grade of inflammation was determined daily on a scale from 0 to 4 with 0 = normal, 1 = light pink, 2 = pink, 3 = red, and 4 = dark red/purple. Inflammation diameter and grade measurements were determined by a researcher blind to the experimental condition of the animal.

Circulating peripheral blood leukocyte enumeration. Baseline blood samples (200 μl + 2 U heparin, Wyeth) were taken from the tail vein, and then both sedentary and run rats were exposed to either IS (previously described) or remained in their home cages. Additional tail vein blood samples were collected immediately (0 h), 1 h, and 2 h after the cessation of IS. White blood cell differentials were measured on a CellDyn 3500 Hematology analyzer. Automated image identification for cell counting was performed. CellDyn 3500 Hematology analyzer settings were based on standard normal ranges for male rats provided by CellDyn. Cell number (K/μl) is reported.

Histological examination of inflammatory site. Inflammatory sites were removed 2 h after IS, placed in Formalin (24 h), and embedded in paraffin. Serial sections (4 microns) were cut and stained with hematoxylin & eosin (H & E). It has been previously documented that the neutrophil is the first immune cell to migrate to the site of bacterial challenge (35). Therefore, neutrophils/area were measured using the NIH (Version 1.60) imaging analysis system. Dermis vascularization or capillary density was measured in H & E sec-
tions. Inflammatory H & E-stained tissues were microscopically examined. Three equally sized dermal areas were examined, and the average number of dermal capillaries per square millimeter of tissue was then calculated. Microscopic measurement of edema at the inflammatory site was also performed using the same procedures. Three equally sized dermal areas were microscopically examined. Representative edematous spaces were identified and measured, and the average area was calculated. An independent pathologist performed the histological examinations and was blind to the animal experimental condition.

Complement function C3-mediated cell lysis. Cardiac blood samples were collected 2 h after IS termination via cardiac puncture. Serum was removed and stored at −20°C. C3 function was determined using a standard cell lysis assay (22). In brief, 10 µl of serum were diluted 1:100 with 1.0 ml of GL-GVB2+ buffer [glucose and gelatin (Sigma), Na-5,5-diethyl barbiturate]. Threefold serial dilution of the stock was prepared. Fifty microliters of C3-depleted serum (Advanced Research Technologies) and 50 µl of sheep erythrocytes plus IgM (2.0 × 10^6; Colorado Serum) were added to 50-µl samples in duplicate. The samples were incubated in a water bath (37°C) for 30 min. Ice-cold saline (1.0 ml, 0.9%) was added to each tube and centrifuged at 2,500 rpm for 5 min. Optical density was determined for each sample (412 nm; Beckman DU 650). For details, please refer to Giclas in Ref. 20.

Statistical analyses. In vivo bacterial measures of inflammatory size and grade and circulating neutrophil numbers were analyzed using 2 (stress vs. no stress) × 2 (run vs. sedentary) with one repeated-measures (time: 0, 1, 2 h) ANOVAs. Fisher protected least significant difference (F-PLSD) post hoc analyses were performed when required. Complement functional activity was analyzed using 2 (stress vs. no stress) × 2 (run vs. sedentary) ANOVAs.

RESULTS

Frewheel running. As previously reported (30), Sprague-Dawley rats in the current studies voluntarily ran in their wheels an average of 3.7 ± 0.2 km/wk and gained slightly less body weight than their sedentary counterparts (run mean = 381 ± 3.7 g; sedentary mean = 400 ± 7.0 g). Direct measurement of individual daily wheel running velocity was not possible.

Bacterial challenge and in vivo inflammation assessment. Inflammation diameter (Fig. 1A) and grade (Fig. 1B) were greatly increased 1 day after subcutaneous injection of E. coli challenge. Stress before E. coli reliably reduced the size [F(1,48) = 41.4, P = 0.0001] and grade [F(1,48) = 33.7, P = 0.0001] of the inflammatory response. Physical activity alone had no effect on inflammation. The effect of stress in the physically active rats was increased, such that physically active and stressed rats had the greatest reduction in bacterial inflammation diameter [F(4,192) = 4.3, P = 0.002] and grade [F(4,192) = 3.7, P = 0.007]. F-PLSD revealed a reliable difference between the impact of stress on inflammation diameter in the sedentary vs. physically active rats on day 1 (P = 0.01) and day 3 (P = 0.004) after stress and E. coli challenge. F-PLSD revealed a reliable difference between the impact of stress on inflammation grade in the sedentary vs. physically active rats on day 3 (P = 0.01) after stress and E. coli challenge.

Circulating peripheral blood leukocyte enumeration. As shown in Fig. 2 and previously reported in the literature, stress alone has a dramatic effect on the total number of circulating white blood cells [F(3,75) = 19.8, P = 0.0001; Fig. 2A], neutrophils [F(3,75) = 17.5, P = 0.0001; Fig. 2B], monocytes [F(3,75) = 23.5, P = 0.0001; Fig. 3A], and lymphocytes [F(3,75) = 8.7, P = 0.0001; Fig. 3B]. Physical activity alone had no effect on the total number of circulating white blood cells, monocytes, or neutrophils. Physical activity did alter baseline numbers of lymphocytes (F-PLSD, P = 0.0008; Fig. 3B), and the number of circulating lymphocytes changed across time [F(3,75) = 167.2, P = 0.0001; Fig. 3B]. Importantly, the effect of stress on the total number of circulating white blood cells [F(3,75) =
Histological examination of inflammatory site. Figure 4 shows the results of the microscopic examination of the inflammatory site. Stress reliably increased the number of neutrophils at the inflammatory site 2 h after the E. coli challenge (Fig. 4A). There was a significant main effect of stress on neutrophil density \([F(1,20) = 34.9, P = 0.0001]\). Importantly, the impact of stress was greatest in physically active rats \([F(1,20) = 14.5, P = 0.0011]\), such that physically active and stressed rats had the greatest number of neutrophils at the inflammatory site 2 h after stress and E. coli challenge. Clearly, neither stress nor exercise alone had any effect on capillary density \((P > 0.05; \text{Fig. 4B})\) or microscopic edema \((P > 0.05; \text{Fig. 4C})\) at the inflammatory site.

**Complement function C3-mediated cell lysis.** Figure 5 shows the effect of stress on C3-mediated cell lysis. Stress reliably increased C3 function 2 h after stressor termination in both sedentary and physically active rats \([F(1,20) = 4.5, P = 0.04]\). Physical activity alone had no effect, and there was no stress condition times physical activity status interaction \((P > 0.05)\).

**DISCUSSION**

The results of the current studies support the hypothesis that regular, moderate, and voluntary physical activity modulates the impact of stress on immunity. Sedentary, stressed rats resolved their inflammation 1–2 days faster and had increased circulating neutrophils compared with their nonstressed, sedentary counterparts. In contrast, physically active, stressed rats resolved their inflammation 3–4 days faster and had in-
Increased circulating and inflammatory site neutrophils compared with their nonstressed counterparts. Importantly, the beneficial impact of stress on bacterial recovery was greater in the physically active, than sedentary, stressed rats. Recovery from bacterial inflammation is a classic in vivo measure of innate immunity. Thus the positive effects of stress on innate immunity are facilitated in physically active organisms.

Importantly, physical activity, per se, had no effect on inflammation. Although previous research demonstrated a direct protective effect of exercise on bacterial illness, most of these studies used forced exercise paradigms and administered infectious agents. Both types of challenges [forced exercise (29) or illness (10)] are known to trigger many features of the stress responses. The data presented here and elsewhere suggest that the activation of the stress responses can facilitate features of innate immunity (8, 9, 11, 12, 19, 24, 37, 39). Thus perhaps the previously reported protective effect of exercise was not directly due to the physical activity but rather indirectly due to the activation of the stress response. Physical activity used in the current studies was moderate intensity, voluntary free-wheel running. We have evidence that this level of physical activity does not trigger a stress response as there are no changes in thymus weight, adrenal weight, corticosterone, antibody response to antigen (17, 30), or activation of brain stress-reactive areas (21).

It is clearly established in the literature that the early stage of inflammation produced following a subcutaneous bacterial challenge involves the migration of neutrophils/monocytes to the site of challenge (2). After migrating into the tissue, the neutrophils/monocytes are stimulated via several different mechanisms (36), and they release a variety of bactericidal substances such as NO, O₂⁻, and inflammatory regulatory proteins (34). We previously reported that exposure to tail shock stress in sedentary animals increases many features of innate immunity, such as macrophage/neutrophil NO (19), proinflammatory cytokines (28, 31), and acute phase proteins (11). The results from the current studies add to the list of effects of tail shock stress in sedentary organisms. Specifically, we found that exposure to tail shock stress in sedentary organisms increases C3 function (Fig. 5) and total circulating white blood cells and neutrophils (Fig. 2, A and B). All of the changes listed above could contribute to the improvements in bacterial inflammatory response reported after tail shock stress.

Importantly, the stress-induced potentiation of innate immunity was most beneficial to physically active rats, because this group resolved their inflammatory response 3–4 days faster than sedentary, stressed rats.

Fig. 5. Adult, male Sprague-Dawley rats (n = 6/group) were allowed to live with either a mobile (Run) or locked (Sedentary) running wheel for 7–8 wk. Rats were then exposed to 100 5-s, 1.6-mA tail shocks (Stress) or no stress and challenged subcutaneously with E. coli (2.5 × 10⁸ CFU). Two hours after bacterial challenge and stress termination, the inflammatory site was removed. Histological examination of neutrophil density (A), capillary density (B), and edema (C) was performed on hematoxylin and eosin-stained sections. Stress × activity interaction, F-PLSD, +P < 0.05.

Fig. 4. Adult, male Sprague-Dawley rats (n = 6/group) were allowed to live with either a mobile (Run) or locked (Sedentary) running wheel for 7–8 wk. Rats were then exposed to 100 5-s, 1.6-mA tail shocks (Stress) or no stress and challenged subcutaneously with E. coli (2.5 × 10⁸ CFU). Two hours after bacterial challenge and stress termination, cardiac blood samples were removed, and C3 lytic function was measured.
Physically active, stressed rats also had the greatest number of neutrophils in the blood (Fig. 2B) and at the site of bacterial challenge (Fig. 4A). Although not conclusive, results from these studies suggest that one way physical activity may facilitate the positive effect of stress on bacterial inflammation resolution is by increasing the mobilization and migration of neutrophils to the site of bacterial challenge during the initial stages of inflammation. It is possible, therefore, that the early (2 h after E. coli) increase in the number of neutrophils at the site of bacterial challenge could benefit bacteria lysis and clearance, and subsequently, contribute to faster inflammation resolution. This idea is supported by the observation that the effect of tail shock on bacterial inflammation diameter is present rather early after challenge. Tail shock stress reduced the size of inflammation 2 h after challenge with benign E. coli (12) and 4 h after challenge with infectious E. coli (6).

There are several feasible alternative explanations for the current results. Physical activity has been previously reported to change dermis vascularization (4). Therefore, one explanation for our results is that physical activity alone increases the density of dermis capillaries. Thus the improvement in stress-induced bacterial resolution in physically active, stressed rats is due to greater circulation to the skin. This is unlikely, however, as we found no change in dermis capillary density (Fig. 4B) in physically active rats. A second explanation for our results is that stress and physical activity could be changing edema at the site of bacterial challenge. We also found no evidence to support this idea. Neither stress nor physical activity changed edema at the inflammatory site (Fig. 4C).

In conclusion, the current studies support the conclusions that acute stress facilitates some aspects of immune function and that physical activity status modulates the impact of stress on immunity. Voluntary freewheel running has been previously reported to modulate the negative effect of stress on immunity, such as natural killer cell cytotoxicity (15) and antigen-specific antibody (17, 30). Thus physical activity can both prevent the negative impact and facilitate the positive impact of stress on immunity. The current research clearly demonstrates one more physiological benefit of maintaining a physically active lifestyle.

REFERENCES


