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Glucocorticoids produced during exercise may be necessary for optimal virus-induced IL-2 and cell proliferation whereas both catecholamines and glucocorticoids may be required for adequate immune defense to viral infection

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Abstract

Prolonged, exhaustive exercise has been associated with impaired immune responsiveness and increased susceptibility to infection. We have shown that one bout of exercise to fatigue followed by viral challenge increases mortality. Stress hormones such as corticosteroids and catecholamines have been suggested as potential mediators of exhaustive exercise-induced immunosuppression. The purpose of this study was to determine whether the administration of pharmacological agents to block the effect of catecholamines or corticosteroids would minimize the immunosuppression associated with this type of exercise. Mice either exercised to fatigue or were exposed to control conditions, and mice received an i.p. injection of either nadolol (β-adrenergic receptor antagonist), RU486 (glucocorticoid type II receptor antagonist), or vehicle. Fifteen minutes post-exercise, mice were exposed to viral infection (Herpes simplex virus; HSV) via an intranasal route, and cells were collected 3 days post-infection. The results showed that exercise suppressed HSV-specific cell proliferation, HSV-specific IL-2, and IFN-γ, but did not alter these same immune parameters when the mitogen ConA was used to stimulate cells. In addition, exercise reduced NK cell cytotoxicity, alveolar cell TNFα, and peritoneal IL-1β, but did not affect IL-10. The pharmacological blockade did not attenuate the exercise-associated immunosuppression. In fact, RU486 treatment exacerbated the exercise-induced decline in HSV-induced IL-2 production and cell proliferation. RU486 and nadolol treatment also tended to decrease IL-10, IFN-γ, TNFα (nadolol only), and IL-1β (RU486 only) in both exercise and control mice, suggesting that stress hormones may be necessary during infection for optimal responsiveness. These findings suggest that suppression of immune defenses during viral infection persists for at least 3 days post-exercise, and stress hormones may be essential for optimal immune defense to viral challenge, rather than detrimental.

1. Introduction

Stress has been associated with immunosuppression and increased susceptibility to infection (Lutgendorf and Constanzo, 2003; Yang and Glaser, 2002). However, the type of stress, duration of stress, coping or adaptive ability of the organism, and the timing of stress exposure in relation to infectious challenge have all been identified as factors in determining whether stress may increase or decrease the susceptibility to infection. Exercise may be considered a type of stress that affects immune responsiveness differently dependent on the duration, intensity, and frequency of the stress (Hoffman-Goetz and Pedersen, 1994). For example, regular, moderate intensity exercise has been associated with reduced susceptibility to infection, reduced severity of infection, and improved antibody responses to vaccine (Cannon and Kluger, 1984; Kohut et al., 2004b; Nieman

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et al., 1993). In contrast, a single prolonged, strenuous exercise bout has been linked with increased susceptibility to infection and reduced immune function (Davis et al., 1997; Nieman et al., 1990; Woods et al., 1999). A better understanding of how exercise might alter immune responsiveness to pathogenic challenge may allow for the development of appropriate guidelines to optimize immunity in the general public, and minimize infection in competitive athletes training at high intensities.

Stress hormones and neuroendocrine factors have been shown to bind to cells of the immune system and influence immune function (Eskandari and Sternberg, 2002; Kohm and Sanders, 2001; Madden, 2003; Riccardi et al., 2002). Numerous studies have demonstrated that by blocking stress hormones (typically catecholamines or corticosteroids), the detrimental effects of stress can be minimized (Dobbs et al., 1993, 1996; Fleshner et al., 1996). Although many studies have reported elevated levels of norepinephrine, epinephrine, and corticosteroids in association with prolonged exercise-induced immunosuppression (reviewed in Pedersen and Hoffman-Goetz, 2000), relatively few studies have attempted to block stress hormones during exercise to examine the role of the stress hormones in modulating immune responsiveness (Bouix et al., 1995; Jonsdottir et al., 2000; Kapasi et al., 2001; Kohut et al., 1998). Even fewer exercise studies have used an infectious disease model to determine whether the manipulation of stress hormones during exercise may impact the immune parameters involved in resistance to or clearance of viral infection (Kohut et al., 1998). Most exercise studies have relied on the use of mitogens, and it is possible that stress-induced alterations of antigen and mitogen responses may differ (Kusnecov and Rabin, 1993). Given that prolonged, exhaustive exercise has been associated with increased susceptibility to upper respiratory infection, a respiratory viral infection model may be highly relevant in terms of studying the role of stress hormones as mediators of exercise-associated declines in the ability to respond optimally to pathogens.

We have previously shown that a single bout of exhaustive exercise followed by intranasal exposure to virus results in increased mortality from infection as compared to either no exercise or moderate exercise (Davis et al., 1997). We have also established that pharmacological blockade of β-adrenergic receptors blunted the exhaustive exercise-induced decline of alveolar macrophage antiviral response using the same model of exercise and upper respiratory viral infection (Kohut et al., 1998). These previous results suggested that catecholamines may mediate the reduction of macrophage antiviral function. Although the macrophage may be considered one of the first lines of defense against viral infection in the lungs, NK cells, and antigen-specific cell mediated responses are also important in clearance of viral infection. One of the goals of the current study was to determine whether stress hormones also mediate the exercise-induced modulation of T cells as well as NK cells. In addition, macrophages may defend against viral infection by the release of TNFα (Rossol-Voth et al., 1991), and macrophages link innate and adaptive immune responses via the release of cytokines such as IL-1β. Therefore, macrophage responses were also evaluated. Dendritic cells derived from both the airways as well as the mediastinal lymph nodes may present HSV antigen to naïve T cells (Belz et al., 2004), although dendritic cell responses were not evaluated in this study. To examine antigen-specific function, a respiratory viral infection model was used. Antigen-specific responses were elicited by incubation of cells from infected mice with the same virus in vitro. We hypothesized that catecholamines, rather than corticosteroids, would mediate exercise-induced immunosuppression similar to our previous observations with alveolar macrophages.

2. Materials and methods

2.1. Mice

Male BALB/cJ mice 6 weeks of age were acclimated to the animal housing facility on 12-h light:dark cycle for 2 weeks prior to experimental intervention. All mice were then acclimated to handling and treadmill exposure. The treadmill exposure was brief (5–10 min) at a very low speed (~4–5 m/min) and was repeated for three consecutive days. After the treadmill acclimation period, mice were randomly assigned to an exercise (EX, $n = 33$) or control group (CON, $n = 18$), and then further divided based on drug treatment group.

2.2. Pharmacological treatment

The non-selective β-adrenergic antagonist, nadolol, was used to determine if the immunomodulatory effects of exercise were mediated by activation of β-adrenergic receptors via catecholamines. Nadolol was injected i.p. at a dose of 5 mg/kg in saline containing 0.2% ethanol within 30 min prior to onset of exercise or control treatment ($n = 11$ EX, $n = 6$ CON). The dose of nadolol was shown in preliminary experiments to blunt the exercise-induced increase in plasma-free fatty acids that is mediated by catecholamines. Therefore, this dose of nadolol produced a significant physiological effect similar to our previously published work (Kohut et al., 1998). To evaluate whether corticosteroids mediated the effects of exercise, the RU486 was dissolved in 40% of 2-hydroxypropyl-β-cyclodextrin in saline, and injected i.p. at 10 mg/kg within 30 min prior to the onset of exercise or control treatment ($n = 11$ EX, $n = 6$ CON). This dose

has been previously shown to block immunosuppressive effects of stress, and has been shown in rats to result in nearly 100% receptors occupancy in the spleen (Fleshner et al., 1996). We did not evaluate receptor occupancy in this study, and we did not determine whether RU486 demonstrated any agonist properties at the dose used in mice as has been suggested in some studies at higher doses (Gruuol and Altschmied, 1993; Whelan et al., 1995). The dose used in our study has been used in another rodent model (rats), and in rats, this dose was thought to have essentially no agonist properties (Fleshner et al., 1996). We did not assess a physiological measure of drug effectiveness and this is a limitation of this study. Vehicle-treated mice ($n = 11$ EX, $n = 6$ CON) received an injection of saline dissolved in 40% of 2-hydroxypropyl-β-cyclodextrin in saline plus 0.2% ethanol.

In pilot experiments, the exercise protocol used in this study was found to result in a significant increase in plasma corticosterone with the mean ± SD as follows: 251.5 ± 151.8 ng/ml for exercise-treated mice as compared to 22.1 ± 17.8 ng/ml for control mice. In addition, the exercise produced physiological results consistent with elevated plasma catecholamines, i.e., increased plasma-free fatty acids (1.10 ± .03 mmol/L in exercise as compared to 0.31 ± 0.02 mmol/L in control mice as mean ± SE). A very similar exercise protocol in a separate study was found to significantly increase plasma norepinephrine and epinephrine (Marra et al., 2005). Also, in pilot experiments, the percentage of $CD4^+$, $CD8^+$ or $SIgM^+$ cells in the spleen, and β1β2-adrenergic receptor density was not different between the exercise and control groups, although the total number of lymphocytes and neutrophils did decrease in the spleen (but not when expressed as lymphocyte per milligram of spleen).

2.3. Exercise treatment

Exercise mice ran on the treadmill at gradually increasing speeds until fatigue. Fatigue was defined as the point at which mice were not able to keep pace with the treadmill belt. Electric shock was never used in the protocol. As mice neared fatigue, if a gentle tap on the tail of the mouse failed to elicit running movement forwards on the treadmill, then the mouse was considered to have reached fatigue and was removed from the treadmill. The treadmill speed began at 11.5 m/min and was increased by 4–6 m/min every 25 min. The maximum speed reached was 42 m/min at 2 h and 30 min of exercise. The exercise began during the last hour of the dark cycle. Control mice were placed in lanes on the treadmill prior to the exercise session. During the exercise session, control mice were kept in Plexiglas boxes next to the treadmill to control for stresses associated with treadmill noise.

2.4. Infection protocol and virus preparation

Within 10–15 min after reaching fatigue, exercise mice were infected with 5×10^3 plaque forming units (PFU) of Herpes Simplex Virus Type I Patton strain (HSV-1) in 50 μl of sterile saline via an intranasal route. Control mice were removed from the treadmill area and infected after a similar time of exposure to the treadmill environment. This dose of virus was shown in pilot experiments to result in a mortality rate of approximately 10% (LD_{10}). The virus had been propagated on Vero cells and the titer was determined by standard plaque assay. The virus was stored at −80 °C and contained 1.75×10^7 PFU/ml. After infection, all mice were returned to their home cages and did not exercise. Euthanasia was performed on mice 72 h post-infection.

2.5. Cell collection

Mice were euthanized by CO_2. Peritoneal lavage was performed using 5 ml of cold Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) to collect peritoneal macrophages. Cells were washed twice in HBSS and counted using a Coulter Counter (Coulter Electronics, Hialeah, FL). Peritoneal cells were adjusted to 1×10^6 cells/ml in complete RPMI media (CRPMI; RPMI 1640, Sigma Chemical, St. Louis, MO; 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mmol/L glutamine, and 10% fetal bovine serum, all from Gibco-BRL, Grand Island, NY). Spleens were removed and dissociated using a lab blender (Tekmar, Cincinnati, OH). Cell suspensions were passed through sterile nylon mesh to remove clumps of tissue. Red cells were lysed with Tris–ammonium chloride and washed two times in CRPMI. Cells were counted and adjusted to 5×10^6 cells/ml in CRPMI. Alveolar macrophages were obtained by transtracheal lavage of the lungs with 5–10 ml of cold HBSS. Red cells were lysed with Tris–ammonium chloride and remaining cells were washed two times in CRPMI. Cells were adjusted to a concentration of 5×10^5 cells/ml in CRPMI. All cells were plated in 24-well plates. Spleen cells were plated at 1 ml/well whereas alveolar and peritoneal cells were plated at a volume of 0.5 ml/well. To isolate adherent cell populations, peritoneal and alveolar cell cultures were incubated at 37 °C for 2 h. After 2 h, wells were washed gently with prewarmed (37 °C) CRPMI.

2.6. In vitro activation of cell cultures

To determine whether antigen as compared to mitogen activation is altered by exercise and drug treatment, spleen cells were cultured with either the mitogen Concanavalin A (Sigma Chemical, St. Louis) at 5 μg/ml, with the viral antigen HSV-1 at a dose of 5 PFU/cell,

or media alone. As expected, both mitogen-induced and HSV-induced cytokine (IL-2, IFN-γ, and IL-10) production were significantly greater than the amount of cytokine produced in wells containing only media, and mitogen-induced cytokine production was significantly higher than antigen-induced cytokine secretion. These findings are consistent with previous results from our laboratory (Kohut et al., 2004a). The adherent alveolar and peritoneal cell populations were activated by the addition of lipopolysaccharide (LPS) at 100 ng/ml.

2.7. Cell proliferation in response to antigen (HSV) or mitogen ConA in vitro

Spleen cells from each mouse were washed twice and resuspended in supplemented RPMI media as described above. Cells were adjusted to 5×10^6 cells/ml and 100 μl was added to each well of a 96-well tissue culture plate (Becton–Dickinson Labware). HSV-1 (antigen) wells contained inactivated HSV-1 at 2.5×10^7 PFU/ml (5 PFU/cell). Mitogen wells contained Concanavalin A at a concentration of 5 μg/ml. Control wells also contained 5×10^6 cells/ml and 1 ml of media without inactivated HSV-1. HSV-1, ConA or media alone was added to triplicate wells containing cells from each mouse. Plates were incubated at 37 °C in 5% CO_2 for 72 h. For the last 4 h of incubation, 10 μl of 5.0 mg/ml of MTT in PBS was added. Four hours later, 100 μl of 0.04 N HCl in isopropanolol was added to dissolve the colored precipitate. Absorbance was read at a dual wavelength of 570 and 630 using an automated plate reader (Bio-Rad, Hercules, CA).

2.8. Cytokine assays

Cytokine assays were performed by ELISA on supernatants from HSV or ConA-stimulated spleen cells. Supernatants for cytokine determination were collected from spleen cells at 24, 48, and 72 h of culture. IL-2, IFN-γ, and IL-10 that was present in supernatants of cells incubated with HSV, mitogen, or media alone were determined by ELISA using monoclonal antibodies and the protocol supplied by BD-PharMingen (San Diego, CA). Cytokines were measured just prior to the peak time point in vitro as determined in preliminary studies. IL-2 and IL-10 were measured after 48 h of incubation with antigen or mitogen, whereas IFN-γ was measured at 72 h of incubation. ConA-induced IL-2 and HSV-induced IFN-γ were also assessed at an earlier time point in vitro as the response was gradually increasing (IL-2 at 24 h and IFN-γ at 48 h), whereas an additional measure of ConA-induced IL-10 was made at the actual peak of the response (72 h). Insufficient cells were available to measure all cytokines in response to both antigen and mitogen at multiple time points in vitro.

2.9. NK cytotoxicity

Spleen cells were adjusted to 1×10^7 cells/ml for the NK cell cytotoxicity assay. YAC-1 cells were used as the target cells in this assay. Briefly, 1.6×10^6 target cells were labeled with 500 μCi [^{51}Cr]sodium chromate for 1 h at 37 °C. Cells were then washed three times, adjusted to a concentration of 1×10^4 cells/ml, and 100 μl was added to the wells of round-bottomed 96-well microtiter plates. One hundred microliters of spleen cells was then added in triplicate at various effector/target (E:T) ratios ranging from 100:1 to 25:1. Total ^{51}Cr release was measured in wells with 100 μl of target cells plus 100 μl of Triton X (detergent). For spontaneous ^{51}Cr release control, 100 μl of supplemented media was added to target cells. Microtiter plates were incubated at 37 °C with 5% CO_2 for 6 h. After incubation, the plates were centrifuged at 400*g* for 10 min and 100 μl of supernatant was removed from each well and counted on a gamma counter. Percent lysis was calculated as

$$\frac{\text{cpm sample} - \text{cpm spontaneous release}}{\text{cpm total release} - \text{cpm spontaneous release}} \times 100.$$

2.10. Statistics

A two-way ANOVA was used to assess the effect of exercise treatment (exercise and control) and drug treatment (nadolol, RU486, vehicle) on the immune variables that were measured. If a significant main effect of exercise was observed or a significant exercise treatment by drug interaction, a follow-up one-way ANOVA was used to assess the effect of drug treatment among the exercised mice. With respect to NK cell cytotoxicity, a three-way ANOVA was used with effector/target dilution as an additional within subjects factor. The time to fatigue during exercise was assessed with a one-way ANOVA.

3. Results

3.1. Exercise performance and results of infection

The results of a one-way ANOVA suggested that there was no difference in the time to fatigue between the three drug treatments [$F(2,31) = 0.735$, $p = .48$]. Mean time to fatigue ± SE for each of the groups was as follows: vehicle 160.3 ± 6.9 min; RU486 151.4 ± 4.9 min; nadolol 159.6 ± 5.6 min. All mice were euthanized at 72 h post-infection, at a time before symptomatic illness (day 4–5 post-infection first onset of symptoms in this infection model).

3.2. Effect of exercise on immune variables

A two-way ANOVA (exercise × drug) was used to analyze the effects of exercise and drug treatment on

immune responsiveness. The results and statistics regarding main effects of exercise treatment are reported in Table 1. The production of Th1 cytokines (IL-2, IFN-γ) in response to antigen (HSV virus) was suppressed by the exercise treatment, and a significant treatment by drug interaction with respect to IL-2 [$F(1,49) = 5.609$, $p = .007$] was found. In contrast, the production of the same Th1 cytokines in response to mitogen (ConA) was not altered by the exercise treatment, but a trend towards an exercise treatment by drug interaction was observed with respect to IL-2 [$F(1,49) = 3.079$, $p = .056$]. Similarly, HSV-induced cell proliferation was significantly lower in the exercise group compared to the control group with a significant exercise treatment by drug interaction [$F(1,49) = 6.458$, $p = .003$], but no exercise differences were found with respect to ConA-induced proliferation. The Th2 cytokine, IL-10, was not altered by exercise in response to HSV or ConA. NK cell cytotoxicity tended to decrease following exercise treatment ($p = .06$). Alveolar and peritoneal cell responses following exercise treatment varied by site and cytokine measured. Exercise suppressed TNFα production by alveolar cells, but did not affect peritoneal cells. In contrast, exercise treatment was associated with reduced IL-1β in peritoneal cells, but did not alter alveolar IL-1β production. In every comparison in which a main effect of exercise was observed, a separate comparison of exercise treatment among only the vehicle-treated mice showed the same results (statistical significance or trend $p < .09$).

3.3. The effect of exercise and drug treatment on Th1 cytokines (IL-2, IFN-γ)

In addition to the main effect of exercise, a significant exercise treatment by drug interaction was observed with respect to HSV-induced IL-2, suggesting that the effect of the drug treatment in the exercised mice was different than the control mice [$F(1,49) = 5.609$, $p = .007$]. Therefore, to test whether drug treatment reversed the suppressive effects of exercise on Th1 cytokine production in response to antigen, a comparison of drug treatment within the treatment groups was evaluated using a one-way ANOVA. Among the exercise group, a trend towards an effect of drug [$F(2,31) = 3.31$ $p = .056$] was observed such that exercised mice receiving the RU486 treatment showed reduced IL-2 as compared to vehicle or nadolol-treated mice, suggesting that RU486 exacerbated the exercise-associated decline in IL-2 (Fig. 1A). In contrast, among the control mice, the results of a one-way ANOVA showed that there was no main effect of drug [$F(2,17) = .064$, $p = .938$], and RU486 treatment did not reduce IL-2. Thus, blockade of stress hormones did not reverse the suppressive effect of exercise on antigen-induced IL-2, and instead, RU486 treatment further exacerbated the exercise-associated decline. With respect to ConA-induced IL-2 production, there was no main effect of exercise or drug treatment (Fig. 1B). However, a trend towards a treatment by drug interaction was observed [$F(1,49) = 3.079$, $p = .056$], and a comparison among exercise only mice tended to show reduced proliferation in vehicle-treated as compared to either drug treatment, but no drug effects were apparent in the control mice. At an earlier time point in vitro (24 h), there was a significant interaction between treatment and drug [$F(1,49) = 4.892$, $p = .012$], although exercise treatment did not decrease ConA-induced IL-2 (data not shown). Instead, a follow-up one-way ANOVA among the exercise mice showed that ConA-induced IL-2 was lower in vehicle-treated mice as compared to either RU486 ($p = .003$) or nadolol ($p = .036$). Therefore, the pattern seen over time in vitro with respect to IL-2 appears to be consistent.

Similarly, drug treatment to block the stress hormones corticosterone or catecholamines did not attenuate the exercise-associated suppression of IFN-γ in

Table 1
Main effects of exercise on immune variables

Immune parameter	Exercise (mean ± SE)	Control (mean ± SE)	*F* statistic	*p* value
ConA proliferation OD	0.235 ± 0.022	0.222 ± 0.029	$F(1,49) = 0.430$	$p = .51$
ConA-induced IL-2 pg/ml	1893.2 ± 18.1	1724.2 ± 22.4	$F(1,49) = 0.149$	$p = .70$
ConA-induced IFNγ pg/ml	356.0 ± 4.2	362.2 ± 3.4	$F(1,49) = 0.240$	$p = .62$
ConA-induced IL-10 pg/ml	1300.3 ± 48.2	1192.0 ± 38.9	$F(1,49) = 2.264$	$p = .14$
HSV proliferation OD	0.220 ± 0.01*	0.277 ± 0.01	$F(1,49) = 6.905$	$p = .01$*
HSV-induced IL-2 pg/ml	44.3 ± 5.3*	66.8 ± 5.2	$F(1,49) = 4.719$	$p = .03$*
HSV-induced IFNγ pg/ml	13.6 ± 2.5*	41.2 ± 8.9	$F(1,49) = 11.72$	$p = .001$*
HSV-induced IL-10 pg/ml	20.1 ± 7.0	11.1 ± 7.9	$F(1,49) = 0.988$	$p = .32$
NK cell cytotoxicity (% lysis)	25.08 ± 1.0+	29.8 ± 1.4	$F(1,48) = 3.553$	$p = .06$+
LPS-induced TNFα alveolar	51.9 ± 9.8*	95.6 ± 22.1	$F(1,49) = 4.814$	$p = .03$*
LPS-induced TNFα peritoneal	18.4 ± 4.4	9.5 ± 2.7	$F(1,49) = 1.371$	$p = .25$
LPS-induced IL-1β alveolar	11.1 ± 2.9	13.8 ± 4.0	$F(1,49) = 0.215$	$p = .65$
LPS-induced IL-1β peritoneal	21.4 ± 8.8*	59.6 ± 14.7	$F(1,49) = 4.62$	$p = .03$*

* Indicates statistically significant at $p < .05$.
\+ Indicates statistical trend at $p < .10$.

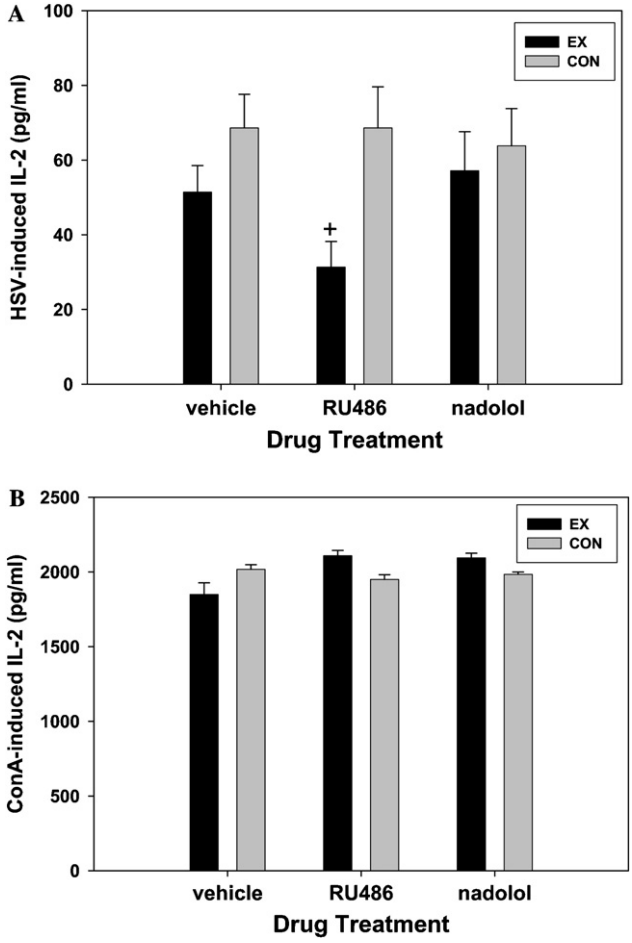

Fig. 1. (A) IL-2 in spleen cell supernatants after 48 h of culture with HSV. CON > EX, main effect ($p = .03$). Exercise by drug interaction ($p = .007$). Effect of drug treatment significant in EX only, such that vehicle and nadolol > RU486 ($p = .05$). (B) IL-2 in spleen cell supernatants after 48 h of culture with ConA. No significant effects of exercise treatment or drug treatment.

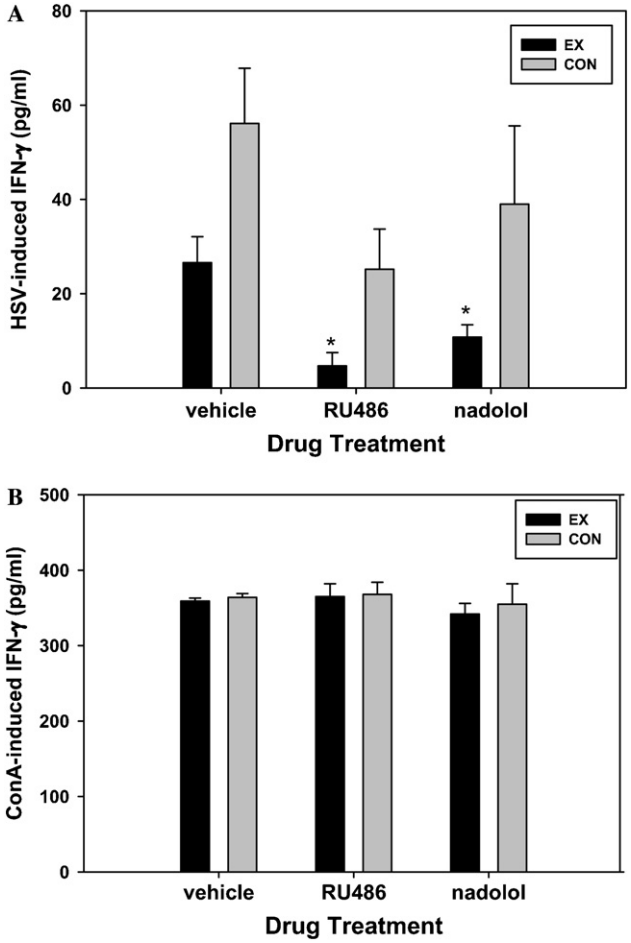

Fig. 2. (A) IFN-γ in spleen cell supernatants after 72 h of culture with HSV. CON > EX, main effect ($p = .001$). Main effect of drug = 0.03 for RU486. Effect of drug treatment significant in EX only, such that vehicle > nadolol and RU486 ($p = .001$). (B) IFN-γ in spleen cell supernatants after 72 h of culture with ConA. No significant effects of exercise treatment or drug treatment.

response to HSV. An exercise treatment by drug interaction was not observed, instead the drug effect tended to be consistent across both groups (main effect of drug, $F(1,49) = 3.872$, $p = .03$), although the magnitude of this effect appeared to be greater in the exercise group. Among the exercise group only, a significant main effect of drug was observed [$F(2,31) = 9.602$, $p = .001$] such that both RU486 and nadolol-treated mice produced less IFN-γ than vehicle-treated mice (Fig. 2A). In contrast, there was no main effect of drug treatment when the control mice were analyzed separately. HSV-induced IFN-γ was also measured at an earlier time point in vitro (48 h, data not shown). The results were similar at this time point showing that exercise treatment tended to reduce IFN-γ (main effect of exercise, $F(1,49) = 3.574$, $p = .06$). However, we did not observe a main effect of drug at this point, and this may be due to the fact that levels of IFN-γ were relatively low and quite variable at this time point. With respect to ConA-induced IFN-γ, exercise or drug treatment did not alter IFN-γ (Fig. 2B).

3.4. HSV or ConA-induced cell proliferation

Although the results suggested a significant main effect of exercise with respect to HSV-induced cell proliferation (Table 1), a significant exercise by drug treatment interaction was also found [$F(2,490) = 8.836$, $p = .001$]. Further post hoc analyses revealed that among the exercise mice, there was a significant main effect of drug treatment [$F(2,31) = 3.361$, $p = .04$] such that the splenocytes from RU486-treated mice proliferated to a lesser extent than vehicle or nadolol-treated mice (Fig. 3A), suggesting that RU486 treatment exacerbated the exercise-associated decline in HSV-induced cell proliferation. With respect to control-treated mice, a slight trend towards an effect of drug treatment was found [$F(2,17) = 2.646$, $p = .10$], with the splenocytes from RU486-treated mice tending

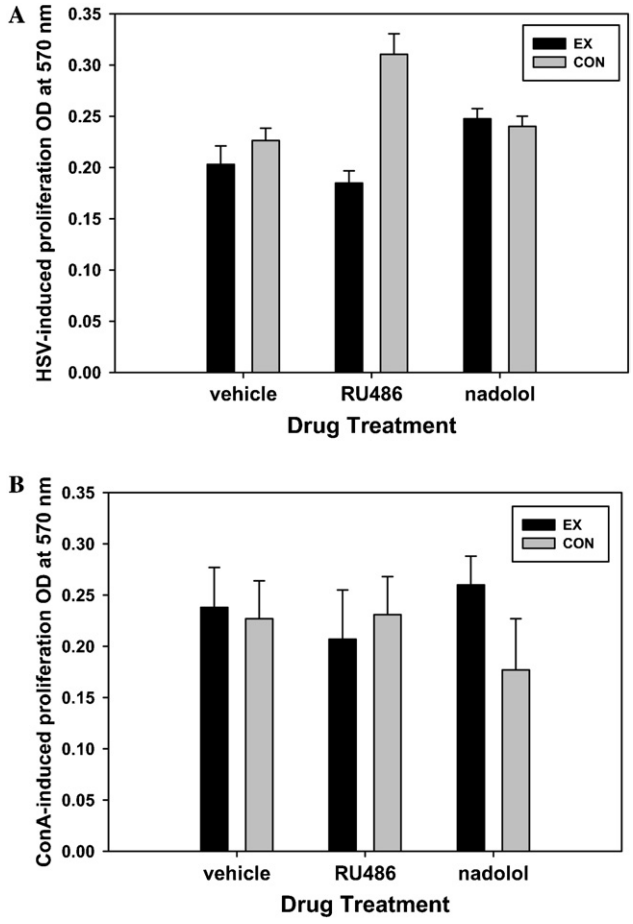

Fig. 3. (A) Spleen cell proliferation in response to HSV. CON > EX, main effect ($p = .01$), although a comparison of vehicle-treated mice only showed a trend toward a significant difference between CON and EX ($p = .08$). Effect of drug treatment in EX only, such that vehicle and nadolol > RU486 ($p = .04$). (B) Spleen cell proliferation in response to ConA. No significant effects of exercise treatment or drug treatment.

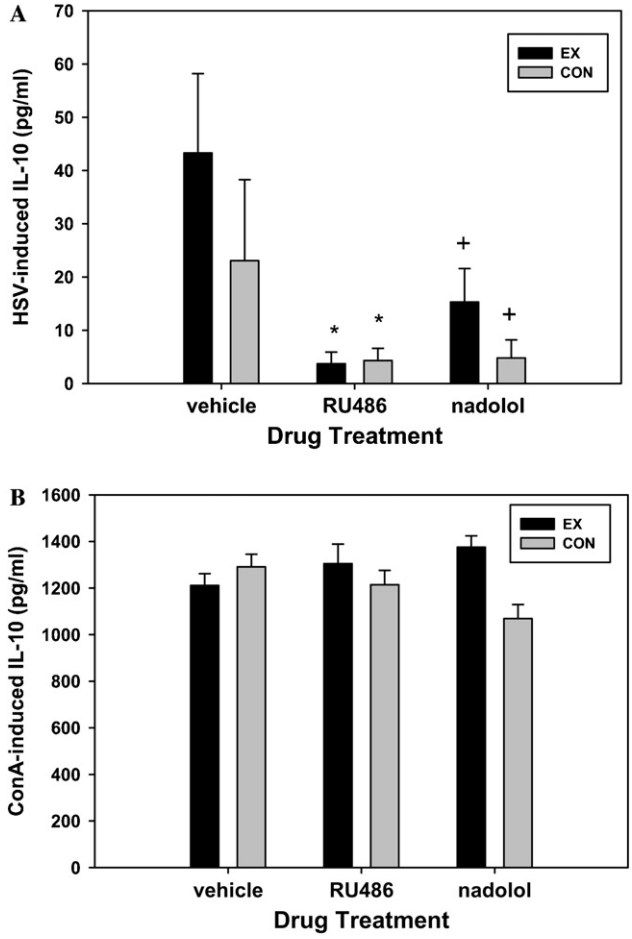

Fig. 4. (A) IL-10 in spleen cell supernatants after 48 h of culture with HSV. No significant effects of exercise treatment was found. Main effect of drug treatment in both EX and CON such that vehicle > RU486 and nadolol ($p = .05$). (B) IL-10 in spleen cell supernatants after 48 h of culture with ConA. No significant effects of exercise treatment or drug treatment.

to demonstrate greater proliferation, a response opposite to that observed in the exercised mice. There was no effect of exercise or drug treatment on ConA-induced proliferation (Fig. 3B), but a trend towards a treatment by drug interaction [$F(1,49) = 2.96$, $p = .062$]. The RU486-treated mice tended to show reduced proliferation in exercised mice, but increased proliferation in control mice.

3.5. Effects of exercise and drug treatment on the Th2 cytokine, IL-10

In contrast to the results observed with Th1 cytokines, exercise did not suppress the production of IL-10 in response to the antigen HSV (Table 1). However, a main effect of drug treatment was observed (Fig. 4A), suggesting that less IL-10 was produced in cells from RU486 and nadolol-treated mice in both the exercise and control group as compared to vehicle-treated mice [$F(2,49) = 3.188$, $p = .05$]. ConA-induced IL-10 production was not significantly affected by exercise or drug treatment and there was not an exercise by drug interaction (Fig. 4B). When ConA-induced IL-10 production was measured at the peak of production (72 h in vitro), again there was no effect of exercise treatment, drug, and no exercise by drug interaction.

3.6. NK cytotoxicity

The results from a three-way ANOVA (exercise treatment by drug by effector/target dilution as repeated measures) suggested that exercise tended to decrease NK cytotoxicity (Table 1). To determine whether drug treatment reversed the suppressive effects of exercise on NK cytotoxicity, a comparison of drug treatment within the exercise group was evaluated using a two-way ANOVA (drug × effector/target dilution as repeated measures) in the exercise-treated mice. These initial results suggested that nadolol tended to reverse

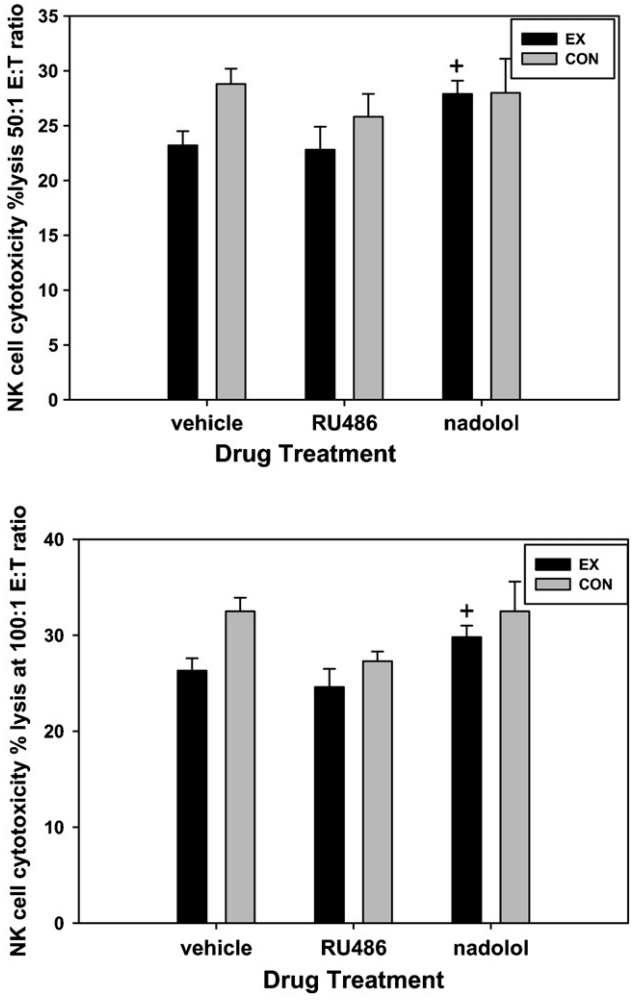

Fig. 5. NK cytotoxicity at 100:1 and 50:1 effector:target ratio. A trend towards CON > EX ($p = .06$). In EX only, trend towards nadolol > vehicle ($p = .06$).

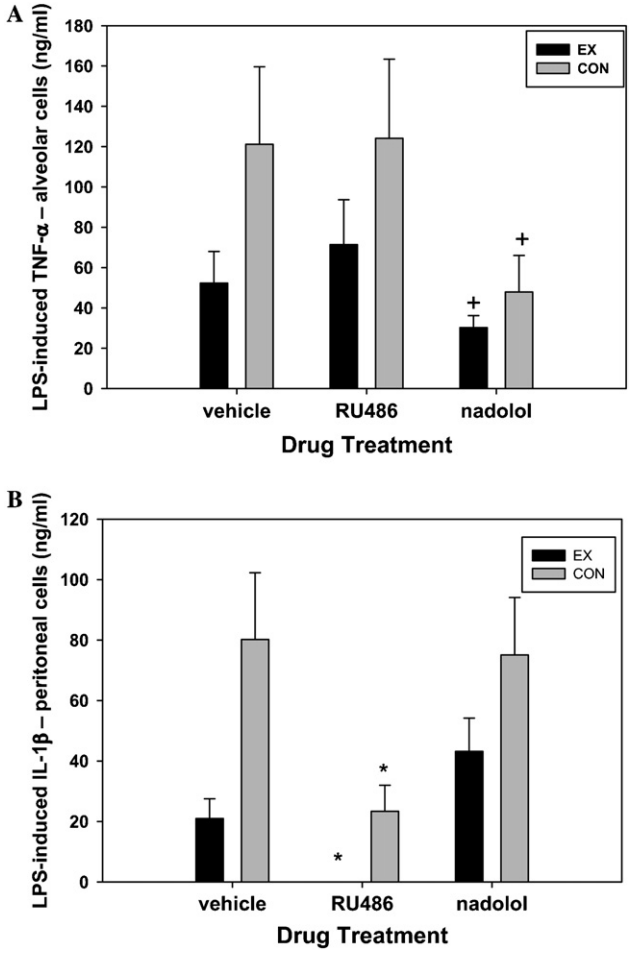

Fig. 6. (A) Alveolar TNFα assessed after 24 h of culture with LPS. CON > EX, main effect ($p = .03$). Trend towards main effect of drug treatment ($p = .06$) such that vehicle > RU486 and nadolol. (B) Peritoneal IL-1β assessed after 24 h of culture with LPS. CON > EX, main effect ($p = .03$). Main effect of drug treatment in both EX and CON such that RU486 < vehicle and nadolol.

the trend of exercise-induced decline in NK cell cytotoxicity [$F(2,31) = 3.626$, $p = .06$]. However, there was also a trend towards a main effect of drug [$F(1,49) = 2.451$, $p = .09$] and no treatment by drug interaction (Fig. 5). Therefore, NK cell cytotoxicity may be independently affected by exercise and drug treatment.

3.7. Alveolar and peritoneal cell (macrophage) function

Cytokine production from the predominately macrophage populations obtained from the lungs and the peritoneal cavity varied by tissue site and exercise treatment. With respect to alveolar cells, exercise suppressed the LPS-stimulated production of TNFα, but had no effect on IL-1β (Table 1). An exercise treatment by drug interaction was not observed. However, a trend towards a main effect of drug treatment was also observed [$F(1,49) = 3.024$, $p = .06$] such that nadolol-treated mice in both the exercise and control groups tended to produce less TNFα, suggesting that catecholamines or corticosteroids do not mediate the exercise-induced decline in TNFα production in alveolar macrophages (Fig. 6A). There was no effect of drug treatment or a drug by exercise treatment interaction with respect to IL-1β.

Peritoneal cell LPS-stimulated release of IL-1β was significantly decreased by exercise treatment, but exercise did not alter TNFα production (Table 1). With respect to both TNFα and IL-1β, there was not a significant exercise treatment by drug interaction. A main effect of drug treatment was found regarding IL-1β such that RU486 treatment was associated with reduced IL-1β in both exercise and control-treated mice [$F(1,49) = 3.721$, $p = .04$], (Fig. 6B).

4. Discussion

The results of these studies: (1) confirmed previous studies suggesting that strenuous exercise may suppress

immune responses, and (2) provide several potential mechanisms by which exhaustive exercise may have resulted in decreased resistance to respiratory viral infection. A decline in antigen-specific Th1 cytokine production (IL-2, IFN-γ), T cell proliferation to antigen, NK cell function, and the alveolar/peritoneal cytokine production that may be relevant to antiviral defense was observed following exhaustive exercise. We previously observed that this exercise protocol reduced HSV-induced IL-2, IFN-γ, and IL-10 at day 2 post-infection, but these cytokines were not different at day 7 post-infection (Kohut et al., 2001). Although IL-10 was not reduced, in this experiment, the response was measured 3 days post-infection and perhaps IL-10 production has been restored at this time point. Our results are generally consistent with others, suggesting that IFN-γ production appears to be most affected by strenuous exercise (Weinstock et al., 1997). Other studies have demonstrated that exercise may suppress some parameters of immune response (for review, refer to Nieman, 1998). However, a major limitation of many studies has been a lack of any association between declining immune response and clinical outcome. We have previously shown that this model of exhaustive exercise followed by controlled exposure to virus results in significantly greater mortality than either moderate exercise or no exercise, providing some support for the link between suppressed immune response and outcome of infection (Davis et al., 1997). We did not assess specific cell populations in these experiments, and the possibility that changes observed reflect changes in cell number rather than activity cannot be determined. One recent study has elegantly shown that the actual number of antigen-specific cells producing IFN-γ was reduced 6- to 10-fold following exhaustive exercise at 8 days post-infection (Kapasi et al., 2005), and therefore, it is possible that the changes we observed are related to a reduction in antigen-specific cell expansion. Other recent work has suggested that exhaustive exercise results in lymphocyte apoptosis of intestinal lymphocytes. It appears that catecholamines and/or oxidative stress, rather than glucocorticoids, may mediate the exercise-associated changes in intestinal lymphocyte apoptosis (Marra and Hoffman-Goetz, 2004; Quadrilatero et al., 2005) and perhaps apoptosis played a role in our viral infection model. Although we cannot determine whether cell number or cell functional changes altered cytokine level, the overall effect was a decline in the total amount of cytokine produced in a given tissue (spleen, lung, or peritoneal cavity).

One interesting aspect of our results was the lack of any exercise-associated effect on ConA-induced responses, whereas HSV-elicited responses tended to be altered by exercise. ConA-induced responses were not affected by drug treatment with the exception of a trend towards increased IL-2 in response to both RU486 and nadolol (a trend opposite to that observed with antigen). To date, much of the literature on strenuous exercise and immune responses has relied on mitogens. Other studies in mice have suggested that an acute bout of strenuous exercise may suppress splenic lymphocyte proliferation (Hoffman-Goetz et al., 1986, 1988), although a time course to determine how long this suppression persists has not been well established. We did not observe a decrease in mitogen-induced proliferation, but proliferation was measured 72 h post-exercise rather than immediately post-exercise as in the studies by Hoffmat-Goetz et al. Human studies use peripheral blood mononuclear cells, and the results from these studies show a slight decline in mitogen-induced proliferation and Th1 cytokine production immediately post-exercise that generally returns to baseline within several hours of recovery (Nielsen and Pedersen, 1997; Weinstock et al., 1997). In contrast, our results suggested that exercise-induced alterations of antigen-specific cells may have persisted for a longer period of time (up to 3 days) post-exercise, one likely explanation for decreased resistance to infection, although our previous work suggested that by 7 days post-exercise there was not a difference in antigen-specific responses. However, the magnitude of the response elicited by mitogen and antigen was different and it is possible that at suboptimal levels of mitogen, the responses may have been more similar to those observed with antigen. It was also possible that our results reflect a delay in the growth/proliferation of antigen-specific cells, rather than function, such that at 72 h post-exercise, there may have been fewer virus-specific cells present in the spleen.

With respect to stress-hormone-mediated alterations of immune defenses, our findings generally did not confirm our initial hypothesis. We had hypothesized that pharmacological blockade of catecholamines would attenuate the exercise-associated decline of immune function based on our previous work regarding macrophage antiviral defenses during prolonged exercise (Kohut et al., 1998). In addition, our hypothesis was based in part on findings from other studies, suggesting that the immunosuppressive effects of stress involve stress hormones (Dobbs et al., 1993, 1996; Fleshner et al., 1996). However, in this investigation, it appeared that RU486 treatment exacerbated the detrimental effect of exercise stress on HSV-induced IL-2 and HSV-induced cell proliferation, suggesting that stress hormones (i.e., glucocorticoids) may be important for optimal immune defense to viral infection following exhaustive exercise. We also observed that the lack of stress hormones during the early phase of infection tended to impair immune responsiveness in general, regardless of exercise treatment (although the magnitude of this effect tended to be greater in exercised mice). Specifically, the blockade of catecholamines and glucocorticoids impaired HSV-induced IFN-γ and IL-10, whereas RU486 treatment

alone impaired peritoneal cell IL-1β production, and nadolol treatment alone tended to impair alveolar cell TNFα. In our model, it appears that stress hormones may be essential for optimal immune defense to viral challenge, rather than detrimental.

Our results were not consistent with our previous work showing that catecholamine blockade attenuated the exercise-induced suppression, nor with others who showed that stress hormones mediate immunosuppression (Dobbs et al., 1993, 1996). One explanation for this discrepancy may be that some of the earliest antiviral defenses (macrophage antiviral effects and NK activity) may have been impaired by stress-induced catecholamines, but other immune parameters that evolve over time may respond positively to stress hormones given that studies have shown these hormones may be elevated within several days after immune stimulation (Besedovsky and del Rey, 1989; Dunn et al., 1989; Fleshner et al., 2001). It may also be difficult to compare our stress model with that used by Dobb et al. Future studies are needed to sort out the contrasting roles of stress hormones.

The concept that stress hormones may confer protective immune benefits during prolonged strenuous exercise may not conform to other hypotheses, suggesting that increased infection following an acute bout of heavy exercise may result from impaired Th1 type responses due to catecholamines (Steensberg et al., 2001). In fact, several investigators have used carbohydrate supplementation during prolonged exercise in an effort to minimize exercise-induced decline of immune responsiveness thought to be due to elevated stress hormones (Bacurau et al., 2002; Henson et al., 1998; Lancaster et al., 2005). However, the role of stress hormones with respect to the immediate or short term post-exercise cellular response to mitogens may not reflect the impact of stress hormones on the development of antigen-specific responses to viral challenge. In fact, the development of an optimal immune response to antigen challenge may depend on the release of stress hormones, as suggested by our data regarding HSV-induced IL-2 and proliferation.

Several lines of evidence indicate that glucocorticoids or catecholamines are essential for the development of an optimal immune response to antigen. Our unpublished results using the same exercise and infection model with 20 mice per treatment group suggested that pre-exercise blockade of catecholamines via β-adrenergic antagonists resulted in greater mortality from HSV infection than exercise treatment alone (day 12 post-infection survival was 75% among exercised mice, but only 53% among exercised mice treated with β-adrenergic antagonists just prior to exercise). Others have shown that adrenalectomy resulted in greater mortality from murine CMV infection as compared to adrenal intact mice (Ruzek et al., 1999). Our unpublished results with the same HSV infection model also showed that adrenalectomy reduced survival in both exercise and control mice compared to adrenal intact control mice even when corticosterone was replaced at basal levels in the drinking water (day 12 post-infection survival was 100% in adrenal intact control mice, 74% in adrenalectomized control mice, 78% in adrenalectomized exercise mice with corticosterone replacement at basal levels, 78% in adrenalectomized mice with replacement of corticosterone at physiological levels observed during exercise and basal replacement after exercise, and 85% in adrenal intact exercise mice that did ultimately decrease to 74% by day 15 post-infection; $n = 20$ mice per treatment group). In another recent study, glucocorticoids have been shown to be essential for optimal IgM and IgG production in response to KLH. In this study, IgG2a and IFN-γ were also influenced by glucocorticoids, but not IgG1 (Fleshner et al., 2001). It has been known for some time that virus infection can increase activation of the HPA axis and norepinephrine catabolites (Besedovsky and del Rey, 1989; Dunn et al., 1989). Perhaps in addition to the positive immunomodulatory role of endogenous glucocorticoids (Fleshner et al., 2001), a downregulation of certain immune response during infection resulting from glucocorticoid production may protect against potentially harmful immune responses. Glucocorticoids may minimize the ''over-production'' of inflammatory mediators that could result in greater mortality, and in support of this possibility, a specific role for TNFα has been shown in mediating mortality with the murine CMV infection model (Ruzek et al., 1999). With respect to HSV infection, it has been shown that HSV-specific CTL function is compromised in both sympathectomized (depleted of tissue catecholamines) and RU486-treated mice (Leo and Bonneau, 2000), again suggesting a potentially beneficial role of these stress hormones during infection. In addition, norepinephrine release in the spleen is increased during antigen-specific activation of $CD4^+$ Th2 cells and B cells, and the depletion of norepinephrine results in suppressed IgM and IgG production (Kohm and Sanders, 1999; Kohm et al., 2000). Taken together, these studies suggest that stress hormones may be necessary for optimal immune responsiveness.

To understand the potential role of stress hormones in our model of HSV infection, it is important to first review the timing of pharmacological treatment in relation to viral infection. In our model, nadolol and RU486 were administered just prior to exercise. Based on our experimental design, these drugs were administered approximately 2.5–3.5 h before viral infection challenge to both control and exercised mice. The half-life of nadolol in humans has been reported to range from 15.5 to 23 h (Kostis, 1990; Shapiro et al., 1981), whereas the half-life of RU486 ranges from 25 to 30 h (Heikinheimo, 1997). Therefore, both drugs were likely present during the early phase (∼ day 1) of viral infection.

Perhaps catecholamines and/or corticosteroids that may have been released early in infection were not able to bind to cells of the immune system due to pharmacological blockade with nadolol or RU486. One possible consequence was the impairment of optimal immune responses observed in our study, such as the inhibition of HSV-induced IL-10 or IFN-γ in spleen cells, or the decline in TNFα and IL-1β in specific cell populations (suggested by the significant main effect of drug treatment with respect to these cytokines). Although the specific role of each of these cytokines during HSV intranasal infection has not been clearly established, mortality from infection was greater in exercised mice treated with β-adrenergic antagonists or adrenalectomy as compared to vehicle-treated exercised mice (unpublished findings), suggesting that these cytokines may have a protective role.

One can speculate that blockade of hormone receptors directly resulted in suppressed cytokine production, and some in vitro data support this possibility. For purposes of brevity, we will describe our findings specifically with IL-10, but similar examples could be found in the literature regarding TNFα and IL-1β (reviewed by Elenkov and Chrousos, 2002).

With regard to IL-10, it has been shown that $CD4^+$ cells exposed to the synthetic glucocorticoid dexamethasone increased IL-10 mRNA (Ramirez et al., 1996). In our experiment, IL-10 production was suppressed in all mice treated with RU486. Perhaps the glucocorticoids produced early during infection that would normally upregulate IL-10 production were unable to do so due to the RU486 treatment. Similarly, catecholamines may enhance IL-10 release (Elenkov et al., 1996). If the nadolol treatment prevented the catecholamines from binding to Th2 cells, IL-10 production may have been inhibited, a possibility supported by our results. However, the balance between different cytokines and neuroendocrine factors is very complex, and it is also possible that changes in IL-10 may have resulted from changes in other cytokines that regulate IL-10, such as IL-12. For example, β-adrenergic blockade may have enhanced IL-12 release, and the increase in IL-12 may have subsequently downregulated IL-10. The complex interaction between cytokines and stress hormones in this model of infection cannot yet be determined from our experiments. However, it does appear that pharmacological blockade of catecholamines and/or corticosteroids during the early phases of infection or antigen challenge may impair optimal immune responsiveness, similar to findings reported by others (Ruzek et al., 1999).

A slightly different situation was observed with respect to the IL-2 and HSV-induced proliferation that were further suppressed by drug treatment with RU486, but only in the exercised mice. A main effect of drug was not observed and therefore, the possibility that an infection-induced release of stress hormones was blocked, thus resulting in impaired cytokine production, does not fit the data. Instead, our findings have suggested that blocking glucocorticoids during the stress situation (exhaustive exercise) compromised IL-2 and cell proliferation. Thus, glucocorticoids appear to have conferred some protection from the viral infection that followed exercise, rather than suppressing immunity. Others have elegantly demonstrated that stress hormones produced during stress may augment immune response (Dhabhar, 2002), and have suggested that detection of a stressor by the neuroendocrine system may "prepare" the immune system for potential immunological challenges that result from the stressor. In our model, stress did not likely enhance immune response, as suggested by increased mortality. Yet, our data have supported the possibility that stress hormones "prepare" the immune system for subsequent challenge—given that the pharmacological blockade of stress hormones further exacerbated the stress-induced decline in IL-2 and cell proliferation. The mechanisms by which stress hormones can upregulate or prepare cells for subsequent challenges were not determined in our study, but are the focus of our ongoing studies.

Finally, our results indicated that different functions in the same cell population may be differentially altered by exercise. HSV-induced IL-2 and IFN-γ declines in the exercise group, but IL-10 was not affected at this time point. Also, alveolar cell TNFα production declined in response to exhaustive exercise, whereas alveolar cell production of IL-1β was not compromised. Similarly, IL-1β production by peritoneal cells was attenuated among exercised mice, but TNFα was not affected. Although these opposing effects may appear incongruent, we and others have observed that macrophage functions in response to different activators or factors such as aging differs by tissue source (Han et al., 1995; Kohut et al., 2004a,b,c; Shimada and Ito, 1996). In our model of infection, at the time of tissue collection, the extent of viral replication was likely much higher in alveolar cells as compared to peritoneal cells given that the virus had been introduced via an intranasal route. As a result, the response of the alveolar macrophages may have been directed primarily towards antiviral defense involving TNFα (Rossol-Voth et al., 1991), whereas the effects of exercise stress on the macrophages in the peritoneal cavity may have varied, due to a different stage of cell activation. It has also been shown that the interaction between infection and stress hormones could have a synergistic effect on the production of inflammatory mediators (Chen et al., 1999) and therefore at this early time point in infection, the neuroendocrine-immune responses in the active site of infection (lung) could differ from the neuroendocrine–immune interaction in non-infected tissue (peritoneum). Further research in numerous cell types in various tissues will help to clarify these site specific and functional differences.

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