Exercise effects on IFN-β expression and viral replication in lung macrophages after HSV-1 infection

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Kohut, M. L., J. M. Davis, D. A. Jackson, P. Jani, A. Ghaffar, E. P. Mayer, and D. A. Essig. Exercise effects on IFN-β expression and viral replication in lung macrophages after HSV-1 infection. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L1089–L1094, 1998.—Mice exercised to fatigue and exposed to herpes simplex virus type 1 (HSV-1) exhibit greater mortality than control mice. In this study, we examined lung macrophage resistance to HSV-1 after exercise in terms of both viral replication and interferon (IFN)-β production. We utilized the reverse transcriptase-rapid polymerase chain reaction to measure the IFN-β mRNA content in alveolar macrophages. IFN release was measured with a bioassay, and viral replication within the macrophage was assessed by plaque titration. Exercised (Ex) mice ran on a treadmill until fatigue while control (Con) mice remained in lanes above the treadmill. After exercise, alveolar macrophages were removed and incubated with HSV-1. Alveolar macrophage IFN-β mRNA was greater in Ex than in Con mice. Culture supernatant from infected macrophages showed a higher degree of IFN release and a higher number of infectious viral particles in Ex vs. Con mice. It is likely that the increase in IFN-β mRNA occurs in response to a higher degree of viral replication. These results suggest that macrophages from Ex mice are less resistant to infection with HSV-1.

important antiviral innate immune responses within the respiratory tract mediate local response to viral infection within the lung. It has been suggested that the cytokines tumor necrosis factor (TNF)-α, IFN-α, and IFN-β may mediate antiviral resistance. Prior experiments (25) in our laboratory demonstrated that poly(I-C)-induced antiviral activity in macrophages from the CD-1 strain of mice against HSV-1 (KOS strain) was mediated via IFN-β. In these prior experiments, antibodies to several cytokines [interleukin (IL)-1, TNF-α, IFN-α, IFN-β, and IFN-γ] were added to HSV-1-infected macrophage cultures, and the antibody to IFN-β was found to result in increased CPE, whereas none of the other antibodies had an effect on CPE (25). As a result of these experiments, we chose to focus on the antiviral effects of IFN-β. One possible explanation for the increased CPE observed in macrophages from exercised mice found in our previous experiments is a decrease in the production of IFN.

A few other studies (5, 13, 26, 32) have reported that stressors, including exercise, can alter IFN production. Stress administered within 1 day before infection altered serum IFN levels. However, the amount of IFN present in serum does not necessarily reflect the immediate local response to viral infection within the lung. Instead, increased serum IFN levels at later time
points may reflect the dissemination of virus into the circulation. An assessment of IFN induction by cells located within the respiratory tract may provide information regarding the early immune responses to infection at the initial site of viral infection.

Another potential explanation for the increased CPE previously observed in alveolar macrophages from exercised mice involves a greater and/or more rapid viral replication within the macrophage, perhaps unrelated to IFN-β production. Several studies (1, 2, 16) have reported an increase in viral titers in various tissues after stress (restraint stress, cold stress, isolation stress, and forced swimming), including the spleen, brain, and heart, and also within the footpad after direct injection of HSV-1. It is possible that a similar response occurs within the macrophage.

The purpose of this study was to examine viral replication and IFN-β production in HSV-1-infected alveolar macrophages after prolonged, strenuous exercise. The same model of exercise was used that had previously resulted in increased CPE in HSV-infected macrophages from exercised mice compared with control mice. IFN-β mRNA expression was assessed by the sensitive reverse transcriptase-polymerase chain reaction (RT-RPCR) technique. In addition, an estimation of IFN release into the supernatant of infected macrophages was determined with an IFN bioassay, whereas the degree of viral replication within infected macrophages was assayed by plaque titration on Vero cells. Differences between alveolar macrophage responses from exercised and control mice were compared in each of these experiments.

**METHODS**

Animals. Male CD-1 mice, 26–28 days of age, were purchased from Charles River Laboratories (Raleigh, NC) and acclimated to our animal resource facility for at least 3 days before any experimental manipulation. After this acclimation period, mice were adapted to treadmill running twice a day for 3 consecutive days before any experimental manipulation. Fatigue was defined as the point at which mice failed to maintain pace with the treadmill despite being given mild physical prodding continuously for 5 min (8, 35). Electric shock was never used in these experiments because mice readily respond to a gentle tap on the tail or hindquarters. Fatigue typically occurred in 2.5–3 h. Mice in the Con group were contained in well-ventilated Plexiglas lanes above the treadmill for an equivalent period of time and exposed to similar handling, noise, and treadmill vibration. Con mice were exposed to this similar environment in an attempt to control for potential psychological stresses associated with the noise and vibration of the treadmill. To control for stresses associated with a new environment, we acclimated all of the mice to treadmill running and exposed them to Plexiglas control lanes twice a day for 3 consecutive days before the actual experiment.

Alveolar macrophage collection and preparation. All animals were killed in a bell jar containing ether immediately after reaching fatigue during exercise or at an equivalent period of time in the control Plexiglas lanes. Lungs were removed, and alveolar macrophages were obtained by transtracheal lavage of the lungs with 15–20 ml of physiological medium (11). The preparation of alveolar macrophages before HSV-1 infection has been previously described (8). The total number of cells was counted and plated either in 35 × 10-mm polystyrene petri dishes (mRNA experiments) or in 12-well plates. Nonadherent cells were removed with a washing before HSV-1 infection. In the experiments involving RRPCR, cells from mice in the Ex group were pooled and the cells from the mice in the Con group were pooled due to a limited number of alveolar macrophages per mouse. The RRPCR experiments were repeated three times with pooled cells for a total n of 3. In the other experiments (IFN bioassay and HSV yield assay), cells from individual mice were used.

Virus stocks and infection of macrophages. HSV-1 KOS strain virus was propagated in Vero cells. Stocks of this virus were titrated on Vero cells by a plaque assay and were found to contain 1.2 × 10^6 plaque-forming units (pfu)/ml. Aliquots were stored at −80°C until used. Macrophages remaining adherent to petri dishes were infected with HSV-1 at an infection rate of 7–8 pfu/macroage. At 12 or 48 h postinfection, RNA was isolated from the alveolar macrophages.

Another virus, vesicular stomatitis virus (VSV), used in the IFN bioassay, was propagated in Vero cells. Stocks of virus were titrated on Vero cells by a plaque assay and contained 9 × 10^6 pfu/ml.

RNA isolation. TRI Reagent (Molecular Research Center, Cincinnati, OH) was added directly to petri dishes (1.5 ml) to lyse macrophages. The manufacturer's procedure was followed to obtain an RNA pellet, a procedure based on the method of Chomczynski and Sacchi (7).

cDNA synthesis and RRPCR. cDNA was synthesized with the procedure described by Tan and Weis (33). The sensitive RT-RPCR was used to quantitate the relative amounts of IFN-β mRNA according to the procedure described by Tan and Weis. To ensure that all RNAs were represented equally in the cDNA pool and to prevent nonspecific annealing of the primers, the total RNA was primed with random hexamers and then subjected to RRPCR. Any small differences in the efficiency of reverse transcription were controlled by normalizing the amount of IFN-β mRNA relative to the content of an abundant mRNA present in the macrophage (mouse β-actin).

An Idaho Technology (Idaho Falls, ID) capillary air thermocycler model 1605 was used to perform RRPCR. Preliminary experiments were performed to establish the range of cycles in which a given amount of cDNA (250 ng) would be amplified in a log-linear fashion. This technique has been shown to be quantitative in terms of the relative amounts of amplified cDNA, provided that comparisons are made within the log-linear range of amplification cycles (33). In this range, each subsequent cycle results in exponential amplification relative to the original amount of message present. For IFN-β, 250 ng of cDNA were amplified in a log-linear relationship between ∼35 and 50 cycles. The primer pair β-actin was amplified in a linear range between 14 and 24 cycles with 200 ng of starting cDNA.

RPCR was performed as previously described (10). PCR conditions for IFN-β primer pairs were denaturation, 94°C for 1 s; annealing, 60°C for 1 s; and elongation, 72°C for 15 s. The PCR conditions for β-actin were denaturation, 94°C for
1 s; annealing, 57°C for 1 s; and elongation, 72°C for 7 s. After RPR, the ends of the microcapillaries were scored and cut, and the reaction mixture was quantitatively transferred to a 2-ml centrifuge tube containing 5 µl of stop/loading buffer. The tubes were heated to 95°C in a heat block for 5 min and immediately loaded into the wells of a 6% acrylamide sequencing gel. After electrophoresis, the bands were detected by autoradiographic exposure, usually 12–24 h. Using a duplicate film as a template, we excised the bands from the gel, and the 32P counts per minute (cpm) were determined by liquid scintillation counting.

The change in alveolar macrophage IFN-β mRNA due to exercise (after infection with the HSV-1) was determined in the following manner. The radioactivity (in cpm) in the amplified DNA fragments corresponding to IFN-β mRNA in macrophages obtained from Ex and Con mice was compared in a single gel. To control for experiment-to-experiment variation, the radioactivity (in cpm) in IFN-β DNA fragments was normalized to counts per minute in β-actin, an abundant mRNA present in the macrophage that did not change with exercise. Preliminary experiments that used poly(I-C), a known IFN inducer, were performed to verify the ability of this technique to observe relative differences in IFN-β mRNA.

HSV-1 yield assay (viral replication assessed by plaque titration). Cells obtained from individual mice in Ex or Con groups were plated in 12-well plates. HSV-1 was added (6–8 pfu HSV-1/cell) to alveolar macrophages. After a 3-h incubation at 37°C, cells were washed to remove nonabsorbed virus and incubated for 48 h in 0.5 ml of complete medium (RPMI 1610 containing 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 20 mmol/l of glutamine; Gibco, Grand Island, NY). The supernatants were then collected and titrated by plaque assay on Vero cells as described previously (18).

IFN bioassay. Cells obtained from individual Ex or Con mice were plated in 12-well plates. Diluted HSV-1 (0.5 ml) was added to the macrophages in an amount of 6–8 pfu/cell while some wells were left uninfected. After a 3-h period at 37°C, the cell monolayers were washed, and 0.5 ml of complete medium was added to each well. Fifteen hours later, supernatants were collected and stored at −70°C until further use. The bioassay is based on the principle that even a small amount of IFN (α or β) present in the supernatants obtained from infected macrophages should provide protection from another viral infection. The supernatants were diluted and added to L929 cells in a 96-well microtiter plate 24 h before infection with 500 pfu of another virus, VSV. Wells were gently washed before the addition of VSV. To assess the degree of protection from infection with VSV, the neutral red dye uptake assay (17) was used to measure any CPE resulting from infection with VSV 48 h later. The neutral red dye uptake assay allows one to quantify the degree of CPE. In this assay, the infected cells failed to take up the dye. The titer was defined as the specific dilution of the HSV-infected macrophage supernatant that resulted in a 50% decrease in CPE. In this bioassay, the possibility that macrophage IFN-α production or the production of other cytokines or effector molecules may have conferred some protection on the L929 cells against VSV infection cannot be ruled out. However, prior experiments performed in our laboratory (25) showed that supernatants from HSV-infected macrophages when added to L929 cells protected these cells from infection with VSV, and only antibody to IFN-β was able to abrogate the effect.

Statistics. Group differences (Ex or Con) in IFN titer as determined from the IFN bioassay were assessed with a standard t-test. Group differences (Ex or Con) in plaque-forming units per milliliter as determined from the HSV-1 yield assay were also assessed with a standard t-test. An ANOVA was used to compare run times with fatigue in the three trials of the IFN-β mRNA experiment. In the experiments involving RPR, pooled samples were used in each experiment to obtain sufficient mRNA. Therefore, the experiment was repeated three times for a total n of 3. A two-way ANOVA (group by PCR cycles) was then used to compare IFN-β mRNA.

RESULTS

Ex. The means (±SE) of the run times to fatigue in the three trials of the experiment designed to measure IFN-β mRNA were 181 ± 27.5, 167 ± 26, and 165 ± 24 min. The run time did not significantly differ among the three experiments.

Alveolar macrophage IFN-β induction in response to HSV infection. A representative autoradiogram from the first experiment in which macrophages obtained from Ex and Con mice were exposed to virus for 48 h is shown in Fig. 1A. The autoradiograph showed an increase in the mass of IFN-β cDNA in Ex vs. Con mice amplified in the range of cycles indicated. The experiment designed to assess IFN-β mRNA was performed in amplified IFN-β cDNA fragments (462 bp) were normalized to β-actin (see METHODS). Cycles, PCR cycles. Only values from amplification in log-linear range (35–50 cycles) are plotted.
three times. In trial 1, seven mice per group were used, cells from the mice in each group were pooled, and RNA was harvested from the macrophages after infection with HSV-1. In trials 2 and 3, cells from the mice in each group were also pooled; however, nine mice per group at each time point (12 and 48 h) were used to obtain ample cDNA. The change in IFN-β from Ex vs. Con mice across all three experiments is shown in Fig. 1B. Preliminary experiments have shown that for the mass of cDNA used, the range of PCR cycles (35–50) corresponded to the linear phase of amplification when IFN-β cDNA counts per minute were converted to a log scale (see METHODS). A two-way ANOVA (group by PCR cycles) was used to analyze the data in Fig. 1B, with log counts per minute in the DNA fragments corresponding to IFN-β mRNA as the dependent variable. A significant main effect for groups and cycles was observed (Ex > Con, P = 0.001), whereas no significant interaction between cycles and groups was found.

IFN bioassay. The RPCR results demonstrated an increase in IFN-β mRNA. To assess whether this translated into a biological effect, we also performed an IFN bioassay. In this assay, supernatant collected from HSV-infected macrophages was added to L929 cells before infection of the cells with VSV. If IFN had been produced by the infected macrophages and was present in the supernatant, the L929 cells preincubated with this supernatant should be protected (fewer CPE) to some degree from infection with VSV. Prior experiments in our laboratory (25) with a similar model suggested that protection was due to production of IFN-β rather than from other cytokines such as IFN-α and TNF-α, but a thorough analysis of all the potential cytokines involved was not performed in this experiment. As seen in Fig. 2, the protection of L929 cells from infection with VSV (fewer CPE) was greater in supernatants produced by infected macrophages from Ex mice compared with Con mice, suggestive of greater IFN production (P < 0.05). The titer was defined as the specific dilution of the HSV-infected macrophage supernatant that resulted in a 50% decrease in CPE.

HSV-1 yield. Supernatant from infected macrophages collected at 48 h postinfection was diluted and added to Vero cells to obtain an estimate of the number of infectious virus particles produced within Ex or Con macrophages. Supernatant from HSV-1-infected Ex macrophages contained 9.2 × 10⁴ pfu/ml, whereas the supernatant obtained from infected Con macrophages contained 6.6 × 10⁴ pfu/ml (Fig. 3; P < 0.05). Clearly, a greater degree of infectious viral particles was released into the culture supernatant in Ex, suggestive of greater viral replication within these macrophages.

DISCUSSION

The results from these experiments demonstrate greater IFN-β mRNA expression and IFN production in HSV-infected alveolar macrophages from Ex mice. This is, to our knowledge, the first study to demonstrate that ex vivo production of a cytokine in response to an infectious agent is altered by exercise. This is also the first study to examine cytokine expression in response to exercise and viral infection in a tissue-specific site. The biological significance of alterations in cytokine production may be difficult to interpret alone, and, therefore, we also examined tissue-specific functional responses to viral infection. We observed a greater degree of viral replication within alveolar macrophages from Ex mice. This observation suggests that alveolar macrophages from Ex mice were infected to a greater degree than those from Con mice, and it is likely that the observed increase in IFN-β mRNA in Ex mice occurred in response to the greater degree of viral replication.

Fig. 2. Supernatant obtained from macrophages infected with herpes simplex virus (HSV)-1 for 15 h. Titer is equivalent to specific dilution of supernatant, resulting in a 50% reduction in cytopathic effects (CPE). *Ex > Con at P < 0.05.

Fig. 3. Number of plaque-forming units (pfu)/ml obtained from supernatant of HSV-1-infected alveolar macrophages from Ex and Con mice. Supernatants were collected at 48 h postinfection. *Significantly higher vs. Con, P < 0.05.
The results from these experiments are consistent with findings from previous experiments by our laboratory (8, 15). In a prior series of experiments, we showed that alveolar macrophages obtained from mice exercised to fatigue (identical Ex protocol) and infected with HSV in vitro exhibited greater CPE compared with those from Con mice, suggestive of a decrease in the ability to defend against HSV-1 infection. The greater CPE most likely resulted from an impairment of macrophage intrinsic antiviral resistance. Our current findings of greater viral replication in macrophages from Ex mice are consistent with the observation of increased CPE in macrophages from Ex mice found in our previous experiments. The greater expression of IFN-β mRNA may have occurred in response to the greater degree of viral replication. In our earlier experiments, we also demonstrated that the identical Ex protocol, followed by HSV infection through an intranasal route in vivo, resulted in a higher rate of morbidity and mortality compared with Con mice. It is possible that the increased rate of morbidity and mortality in Ex mice resulted from the decrease in alveolar macrophage intrinsic antiviral activity. In fact, in vivo resistance to HSV infection has been correlated with macrophage antiviral function (19, 29). The importance of the alveolar macrophage in terms of resistance to intranasal HSV infection was suggested in other experiments. These experiments showed that prior activation of alveolar macrophages decreased mortality to HSV-1 intranasal infection (11). Considering that one of the roles of the alveolar macrophage in viral infection is to limit the spread of the virus, a decrease in this intrinsic antiviral function may have contributed to the exercise-induced increase in mortality.

The findings observed in our most recent experiments (increased IFN-β and a greater degree of viral replication in macrophages from Ex mice compared with those from Con mice) illustrate the importance of measuring functional aspects of immune response in addition to cytokine production. Without this biological measure of viral replication, an assumption may have been made that elevated IFN-β production was suggestive of enhanced resistance to viral infection. Instead, it is likely that the induction of IFN-β mRNA occurs in response to the greater degree of infection in macrophages from Ex mice. Taken together, the findings are consistent with the concept that exercise and infection with HSV leads to greater CPE, likely due to increased viral replication within macrophages, resulting in elevated production of IFN.

Whereas, to our knowledge, HSV-1 replication has not been measured within infected alveolar macrophages after exercise or any other type of physiological stress, several studies reported higher titers of virus after other stressors. For example, restraint stress resulted in higher titers of HSV-1 in the footpads of infected mice, and Bonneau et al. (2) attributed this to a decrease in the cytotoxic T-cell response. Increased HSV-1 titers were also observed after footshock stress (16). Another group of investigators (12) utilizing forced swimming as a stressor observed an augmented viral replication within the hearts of mice exposed to coxsackievirus. Considering the findings from these studies, it is not surprising that we observed a slightly higher production of infectious viral particles in macrophages from mice exercised to fatigue than in those from Con mice.

Although this study did not attempt to establish how exercise resulted in elevated viral replication and enhanced IFN production, it is possible that the exercise-induced elevation in circulating catecholamines may have contributed to the immunosuppression of macrophage antiviral function. One of our prior studies (15) that used the identical exercise paradigm demonstrated that administration of the beta blocker propranolol before exercise eliminated the exercise-induced decrease in macrophage-intrinsic antiviral function. Other investigators (9) have provided evidence that catecholamines were involved in stress-induced immunosuppression to HSV. Several in vitro experiments also demonstrated a modulating role for catecholamines in viral infection. One study (14) revealed that epinephrine added to macrophages in culture inhibited macrophage-extrinsic antiviral function. Macrophage function can be altered by catecholamines and other β-adrenergic agonists, and these appear to exert their effects through elevations in cAMP (4). In terms of viral infection, cAMP has been shown to increase the spread of virus from cell to cell, increase adherence of infected cells to uninfected cells, and increase viral replication (22). It is possible that the induced elevation in circulating catecholamines, leading to changes in cellular cAMP that eventually result in greater rates of infection.

The mechanisms mediating macrophage-intrinsic antiviral function in this model are not clear. In general, cellular resistance to viral infection involves the production of IFN, which can impair various steps of viral replication (30). The production of IFN-β has been shown to mediate antiviral activity to HSV-1 in peritoneal macrophages (25). We initially thought that a decrease in IFN production may explain the increased CPE in response to exercise. However, in this study, the production of IFN-β did not correlate with resistance to HSV-1 infection. Other investigators (31) have reported that macrophage-intrinsic resistance to HSV-1 may not involve IFN production. There have also been reports (6, 34) that other factors such as TNF-α or nitric oxide may mediate antiviral activity. It is possible that these factors and/or other cytokines may exhibit antiviral function in our model of HSV-1 infection of the alveolar macrophage. Furthermore, it is possible that exercise-induced alterations in the ability of the macrophage to produce TNF-α, nitric oxide, or other cytokines in response to infection contributed to the decrease in macrophage antiviral activity. In fact, a recent investigator (23) showed that acute stress can alter alveolar macrophage cytokine and nitric oxide production. Finally, it has been shown that elevated cAMP levels and activation of β-adrenergic receptors (which may occur during exercise as a result of increased catecholamines)
inhibit nitric oxide and TNF-α release (3, 4). Recall that, in our previous experiments (15), blockade of β-adrenergic receptors eliminated the exercise-induced suppression in macrophage antiviral function. IfTNF-α, nitric oxide, or perhaps other cytokines exhibit antiviral activity in this model of infection, it is possible that exercise-induced elevations in catecholamines suppress the production of these cytokines, thereby resulting in greater viral infection. Experiments are underway to test this possibility.

In summary, the findings from this study show that IFN-β mRNA is increased in macrophages from Ex mice after HSV-1 infection, most likely in response to the greater degree of viral replication observed in these macrophages. These findings are consistent with previous observations (8, 15) of increased CPE in macrophages. These findings are consistent with previous observations (8, 15) of increased CPE in macrophages. These findings are consistent with previous observations (8, 15) of increased CPE in macrophages.

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