Intracellular mechanisms responsible for exercise-induced suppression of macrophage antigen presentation

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Intracellular mechanisms responsible for exercise-induced suppression of macrophage antigen presentation. J. Appl. Physiol. 88: 804–810, 2000.—In a previous study, we demonstrated that exhaustive exercise suppressed peritoneal macrophage antigen presentation (AP). In this study, we explored the intracellular mechanism(s) responsible for this suppression. Pathogen-free male BALB/c mice (8 ± 2 wk) were randomly assigned to either home cage control (HCC) or exhaustive exercise stress (Exh) treatments for a period of 4 days during induced peritoneal thioglycollate inflammation. Elicited macrophages were harvested, purified, and incubated with chicken ovalbumin (C-Ova, 2.5 and 10 mg/ml) for 18 h. After macrophages were washed, they were cocultured with C-Ova-specific T cells for 48 h at which time the supernates were harvested and analyzed via ELISA for interleukin (IL)-2 as an indicator of macrophage AP. There was no significant (P > 0.05) difference in macrophage AP between cells fixed with paraformaldehyde vs. those that remained unfixed, suggesting that Exh did not affect production of soluble factors influencing macrophage AP (i.e., IL-1, IL-4, PGE2). The ability of macrophages to generate C-Ova immunogenic peptides was analyzed using FITC-labeled C-Ova, which shows fluorescence only when degraded intracellularly. There was a significant (−20%, P < 0.05) suppression in fluorescence in the Exh compared with HCC, indicating a possible defect in the ability of macrophages from Exh to degrade C-Ova into immunogenic peptides. Macrophages were also incubated with C-Ova immunogenic peptide in a manner identical to that for native C-Ova. We found a similar suppression (−22–38%, P < 0.05) in macrophage AP using a C-Ova peptide when compared with native C-Ova in the Exh group, indicating reduced major histocompatibility complex (MHC) II loading and/or C-Ova-MHC II complex cell surface expression. In conclusion, these data indicate an intracellular defect in the macrophage antigen processing pathway induced by Exh.

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pressed AP. Therefore, the purpose of this study was to further explore the intracellular mechanism(s) responsible for the suppression in peritoneal macrophage AP observed following exhaustive exercise stress (Exh) in mice.

METHODS

Animals. BALB/cByJ inbred male mice (8 ± 2 wk) were used in this study because of the MHC compatibility (I-A^d) with the T cell hybridoma employed and our previous experience with this strain. Mice were housed 3–5 per cage (12 × 17 × 28 cm) in a specific pathogen-free animal containment facility on a 12:12-h light-dark cycle (0600–1800 light) at 23°C. Mice were provided autoclaved food (8640 Harlan Teklad 22–5 Harlan, Madison, WI) and water ad libitum. All experiments were performed at the beginning of the light cycle (0600–0900), and the animal treatments were approved by the Laboratory Animal Care Advisory Committee at the University of Illinois at Urbana-Champaign and were within National Institutes of Health guidelines.

Exercise protocol. The exercise protocol consisted of treadmill running. This mode of exercise was chosen because exercise intensity and duration can be experimentally manipulated and quantified (unlike voluntary wheels or swimming) and because we have shown that it suppresses AP in macrophages (5). Mice (3–5 per group in ≥3 experiments) were randomly assigned to one of the following two groups: home cage control (HCC) or Exh. The HCC group served as temporal controls and remained sedentary in their cages during the 4-day treatment period. The Exh group exercised for 2.5–3 h at gradually increasing speeds (18–40 m/min) at 5% grade. Electric shock or prodding was never used in these experiments, as the mice ran well without extrinsic motivation. The animals exercised for 4 consecutive days during the time necessary for TG to recruit macrophages to the peritoneal cavity. TG was injected intraperitoneally (1 ml/mouse) on day 1 immediately after the first exercise session. TG was employed as a macrophage-eliciting agent for two reasons: 1) its action resembled inflammation, making it possible to study the effects of exercise stress on an inflammatory response, and 2) it provided increased numbers of macrophages necessary to perform the experiments and other assays.

Tissue collection and processing. Immediately after the final exercise session (day 4), the mice were killed by rapid CO2 asphyxiation and weighed; the tissues were extracted and processed. The peritoneal cavity was aseptically lavaged with 10 ml of RPMI 1640 (GIBCO, Grand Island, NY) containing 1 U/ml of sterile heparin to obtain peritoneal exudate cells (PECs). PECs from 3–5 mice were pooled in each experiment to obtain enough cells for analysis, and each experiment was performed multiple (≥3 times) experiments. The PECs were washed (190 g, 5 min, 4°C) twice; counted, and stained with trypan blue and were always >95% viable. These cells were adjusted to a concentration of 2 × 10^6 cells/ml in RPMI 1640 containing 5% heat-inactivated, low-endotoxin (<0.01 ng/ml) fetal bovine serum (FBS, Sigma Chemical, St. Louis, MO), 10^{-5} M 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 U/ml), and glutamine (20 mM) for use in the macrophage AP and flow cytometric analysis.

Macrophage AP assay. The macrophage AP protocol was adapted from published work (14, 15) and described in detail in our previous study (5). Briefly, 4 × 10^5 PECs/well were plated onto 96-well flat bottom microtiter plates and incubated at 37°C, 5% CO2-95% air, and 95% humidity for 3 h to allow the macrophages to adhere to the plate. The plates were then washed four times with RPMI 1640 to remove all nonadherent cells, and the macrophage number was quantified as previously described (5). After this, optimal (10 mg/ml) or suboptimal (2.5 mg/ml) concentrations of chicken ovalbumin (C-Ova; Sigma Chemical) and optimal (10 μg/ml) or suboptimal (2.5 μg/ml) of C-Ova immunogen peptide (Ile-Ser-Gly-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arc; Biotech Peptide Synthesis Laboratory, University of Illinois) (5) were added to the plates. The plates were incubated 18 h and washed four times with RPMI 1640 to remove any residual C-Ova or peptide, and 2 × 10^5 hybridoma T cells were added per well. The plates were then incubated at 37°C, 5% CO2-95% air, and 95% humidity for 48 h after which time the supernatants were harvested and stored at −80°C until determination of interleukin (IL)-2.

The hybridoma T cells (AO-40.10AG1) were created and kindly provided by Dr. Philippa Marrack at the National Jewish Hospital and Research Center (Denver, CO). Although the hybridoma will grow without any stimulation, it does not produce IL-2 without the presentation of C-Ova by an APC such as a macrophage (15). Therefore, IL-2 production in this in vitro system is directly proportional to macrophage AP. The T-cell hybridoma line was maintained in medium consisting of RPMI 1640 with 10% FBS, 10^{-5} M 2-mercaptoethanol, and 100 U/ml penicillin-streptomycin-L-glutamate at 37°C with 5% humidified CO2. The cells were seeded at a density of 1 × 10^5 cells/ml and were passed every 3 days. Cells were used in all experiments on the third day of growth. Frozen lots were rederived monthly, and all experiments used cells that had grown for the same amount of time to ensure accurate and reliable results.

IL-2 ELISA. An IL-2 ELISA was developed using an IL-2 anti-cytokine capture antibody (Ab; Pharmingen, San Diego, CA) and a biotinylated IL-2 anti-cytokine detection Ab (Pharmingen). Briefly, the capture Ab was diluted to 2 μg/ml in coating buffer, and 50 μl/well were added to the ELISA plates and incubated at 4°C overnight. The plates were blocked with PBS containing 10% FBS to reduce nonspecific binding. Serial dilutions of IL-2 standards (Sigma Chemical; 0.0–2,000 pg/ml) and the macrophage AP supernatants were added to the appropriate wells and incubated overnight at 4°C. After the second incubation, 100 μl of 1 μg/ml detection Ab were added and the plates were incubated at room temperature for 45 min. After this incubation, 100 μl of 1.25 μg/ml streptavidin-peroxidase (Sigma Chemical) were added, and the plates were incubated at room temperature for 30 min. Finally, 100 μl of 2.2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical) substrate were added, and the plates were allowed to develop at room temperature for 60 min. Color change was quantified by light absorbency on a microplate reader at 405 nm.

Macrophage autocrine/paracrine regulation of AP. Macrophages have been found to produce soluble factors in vitro that could potentially suppress T cell IL-2 secretion and account for the Exh-induced suppression in macrophage AP. An exercise-induced increase in the production of IL-4, PGE2, nitric oxide (NO), or transforming growth factor-β (TGF-β) or a suppression of IL-1 or IL-12 could lead to reduced T-cell IL-2 production (28). These factors could act directly on the macrophage (autocrine) or on the T cell (paracrine) to suppress IL-2 secretion (28). We tested this possibility by incubating macrophages with C-Ova for 4 h and then fixing the macrophages with 0.5% paraformaldehyde. The fixation procedure renders macrophages incapable of producing any soluble factor during the 48 h coculture that would influence the AP assay (i.e., T cell IL-2 production). It has been previously shown that macrophages present antigen even when fixed, assuming they have been cultured with antigen
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for at least 3 h, the minimum time necessary to complete the process (13).

Macrophage antigenic peptide generation. Macrophage antigenic peptide generation was measured by utilizing C-Ova labeled with FITC. FITC (isomer 1) was conjugated to lysine residues on native C-Ova protein similar to that previously described for BSA by Voss and colleagues (29). The FITC-labeled C-Ova was followed kinetically through the AP process to assess the intracellular breakdown of the three-dimensional structure of C-Ova. This technique allowed the measurement of antigenic peptide generation for comparison between the HCC and Exh groups. The fluorescence of FITC conjugated to C-Ova is quenched when C-Ova is in its native three-dimensional configuration, and it will not fluoresce until it has been processed into antigenic peptides (29). Thus the mean fluorescent intensity (MFI) of the intracellular FITC-labeled C-Ova is directly proportional to the amount of antigenic peptide generation. In our experiments, the intracellular FITC MFI of ~10,000 macrophages was recorded via analysis on a Coulter XL-MCL (Coulter, Miami, FL) flow cytometer. Adherence-purified peritoneal macrophages from either HCC or Exh were plated at 5 × 10^5 cells per 10-mm petri dish in RPMI 1640 with 10% FBS. The macrophages were then incubated for 30 min before the addition of 10 mg/ml of FITC-labeled C-Ova and then incubated for either 2 or 4 h. These times were chosen on the basis of preliminary kinetic experiments and work using FITC-labeled BSA (29).

After the incubation, the macrophages were washed twice with RPMI 1640 to reduce extracellular fluorescence and then scraped off the petri dishes with Teflon scrapers. These cells were washed and resuspended in 300 µl of PBS for analysis via flow cytometry for antigenic peptide generation. Cells incubated with unconjugated C-Ova were run in the same experiment to serve as controls.

Data analysis. All data are reported as means ± SE. Significant differences between groups were determined by ANOVA procedures with significance level set at P < 0.05. Student-Newman-Keuls contrast procedures were performed when significant main effects were found.

RESULTS

In a previous study, we found a significant long-lasting (~24 h) reduction in peritoneal macrophage AP after four repeated bouts of Exh (2.5–3 h/daily) in mice (5). This effect was not associated with differences in macrophage number or expression of ICAM-1, B7-2, or total MHC II. In this study, we sought to identify the intracellular mechanisms responsible for this Exh-induced suppression of macrophage AP.

Effects of exercise stress on macrophage autocrine/paracrine regulation of T cell IL-2 production. To determine whether Exh-induced changes in the production of soluble mediators (i.e., IL-4, PGE2, NO, TGF-β, IL-1, IL-12) could account for reduced T cell IL-2 production, macrophages were fixed following a 4-h incubation with C-Ova. T cells were then added to fixed macrophages and cultured for 48 h, and the supernate was assayed for IL-2. Although the fixation lowered the absolute magnitude of IL-2 production, the fixation did not affect the Exh-induced suppression in macrophage AP as measured by T cell IL-2 production (Fig. 1). Because of inherent interassay variability common in bioassays of this type, which makes statistical analysis across experiments difficult, in Fig. 2, we present data from three separately performed experiments as a function of percent change from unfixed or fixed HCC wells. We found a significant (P < 0.05) Exh-induced suppression in both the unfixed (~23.9 ± 2.3% and ~26.2 ± 15.7% for 2.5 and 10 mg C-Ova/ml respectively) and fixed (~23.6 ± 2.2% and ~26.5 ± 5.7% for 2.5 and 10 mg C-Ova/ml, respectively) conditions. On the basis of results from these experiments, we conclude that macrophage production of autocrine or paracrine factors is likely not responsible for the reduction in T-cell IL-2 production seen in response to repeated bouts of Exh. However, these factors do play a role in optimizing T-cell IL-2 production in response to macrophage AP.

Effects of exercise stress on macrophage antigenic peptide generation. To determine whether the ability of macrophages to generate antigenic peptide was compromised, a FITC-labeled C-Ova probe was utilized that would only show fluorescence when the native protein had been enzymatically processed into antigenic peptides in the phagolysosomes of the macrophage (29). Overall, there was significant (P < 0.05) Exh-induced suppression in the ability of macrophages to generate antigenic peptides at the 4-h time point compared with HCC (Fig. 3). In three separate experiments, an average reduction in MFI of ~4.4% and ~17.5% was found in macrophage antigenic peptide generation from that of HCC at 2 and 4 h, respectively. These data indicate that Exh significantly decreases the ability of macrophages to produce antigenic peptides that are essential for AP and the development of a cell-mediated immune response. This reduction in antigenic peptide generation could explain the reduced macrophage AP as measured by T cell IL-2 production following Exh.

Fig. 1. Effects of macrophage fixation on the exhaustive exercise stress (Exh)-induced suppression of macrophage antigen presentation at suboptimal and optimal chicken ovalbumin (C-Ova) doses. Data are from a representative experiment, and data are depicted as means ± SE of duplicate culture wells. *Significant suppression relative to corresponding unfixed or fixed home cage control (HCC), P < 0.05. + Significant suppression in fixed HCC compared with unfixed HCC, P < 0.05.
Effects of exercise stress on macrophage immunogenic peptide presentation. To determine whether C-Ova breakdown into antigenic peptides was the sole mechanism responsible for the reduction in macrophage AP, the ability of macrophages from Exh mice to present the immunogenic portion (amino acid residues 323–339) of the 339-amino acid C-Ova protein was assessed. This immunogenic peptide of C-Ova does not need to be processed to be loaded onto MHC II and expressed as an MHC II-immunogenic peptide complex on the macrophage surface (26). Experiments using this immunogenic peptide allowed the determination of whether antigenic peptide generation was the only step responsible for the diminished AP following Exh.

Data in Fig. 4 demonstrate that Exh significantly (\(P < 0.05\)) reduced macrophage AP by 22–40%, depending on C-Ova peptide concentration. These results were consistent with the previous findings that indicated a 25–34% reduction in the presentation of native C-Ova protein by macrophages (5). These data indicated that antigenic peptide generation was not solely responsible for the decreased macrophage AP following Exh. Therefore, MHC II loading and MHC II-immunogenic peptide complex translocation to the macrophage surface may also be responsible for our findings of reduced macrophage AP following Exh.

**DISCUSSION**

Previously, we demonstrated that Exh suppressed peritoneal macrophage AP (5). This suppression could not be explained by differences in macrophage cell number, adherence, or accessory molecule (i.e., ICAM-1, B7-2, or total MHC II) expression. Likewise, other studies have demonstrated similar reductions (~33–70%) utilizing various types of chronic stress such as long-term ethanol consumption (19, 30), dietary protein deprivation (6, 24), chemical hypotension (9), chronic viral infection (3), HIV (23), and trauma (2). However, none of these studies, including ours, has identified the intracellular mechanism(s) responsible for the suppression AP. Therefore, in this study, we sought to explore the intracellular mechanism(s) responsible for the suppression in peritoneal macrophage AP observed following Exh.
Elevated secretion of PGE$_2$ has been found to be responsible for the suppression in peritoneal macrophage AP in response to the stress of chemically induced hypotension (9). In this report, we present data that rule out the possibility that exercise affected the production of autocrine- or paracrine-soluble factors (i.e., IL-4, PGE$_2$, NO, TGF-$eta$, IL-1, IL-12) that potentially could influence macrophage AP. We did this by fixing macrophages after C-Ova exposure and comparing their ability to present antigen with unfixed cells. The results indicated that in vitro macrophage production of soluble factors was not responsible for the diminished AP following Exh, since the same percentage that was suppression induced by Exh was found in both the fixed and unfixed conditions.

On the basis of these findings, we suspected that the event(s) responsible for the exercise stress-induced reduction in macrophage AP occurred intracellularly. We tested this by assessing the ability of macrophages to generate antigenic peptides, load them onto MHC II, and translocate the MHC II-immunogenic peptide complex to the macrophage cell surface. With the use of FITC-labeled C-Ova (which exhibits fluorescence only when C-Ova is degraded proteolytically into small immunogenic peptides), we demonstrated that Exh reduced the ability of macrophages to generate antigenic peptides. This indicated that the reduction in macrophage AP following exhaustive exercise may be mediated, in part, by a diminished ability of macrophages from exhaustively exercised mice to generate antigenic peptides. In one of the few studies analyzing antigenic peptide generation, Pepin et al. (22) demonstrated that heat shock increased B cell antigenic peptide generation by increasing cathepsin B activity but decreased AP, suggesting a failure in the mechanism of peptide loading onto MHC II molecules (22).

One potential disadvantage of using FITC-labeled C-Ova as an indicator of antigenic breakdown is the independent influence of pH (i.e., acidic pH lowers FITC signal intensity) on fluorescence (12). However, at the low pH range of the phagolysosome (3.5–4.5), there is very little (~5%) change in the FITC signal (12). On the basis of our indirect evidence using this technique and the report of Tsuboi et al. (27) who found that exhaustive exercise increased intralysosomal pH in liver macrophages (27), we suspect that pH may have increased in the phagolysosome in response to Exh. Therefore, any influence that changes in pH might have had would have actually led to an underestimation of the magnitude of the exercise effect. An exercise-induced reduction in phagolysosomal pH could also have reduced the FITC signal independent of antigenic breakdown, but it is unrealistic to believe that phagolysosomal pH would drop much lower than 3.5 in response to exercise, and if it did it would have a minimal effect on FITC signal intensity because this part of the pH vs. FITC signal curve is relatively flat (12). Therefore, although we believe that elevated pH and reduced cathepsin activity may be partly responsible for a reduction in macrophage AP, we cannot say for sure, as we did not directly measure it.

In addition to using FITC-labeled C-Ova as an intracellular probe, we also utilized the immunogenic peptide portion of C-Ova to further explore the extent to which antigenic processing vs. antigenic loading onto MHC II or ferrying to the cell surface may have contributed in reducing macrophage AP. The immunogenic peptide for C-Ova does not require processing to be loaded onto MHC II and expressed as an MHC II-immunogenic peptide complex on the macrophage surface (26). Thus this measurement would reveal whether loading and translocation were also affected by Exh. We found similar exercise stress-induced reductions in macrophage AP when comparing the native C-Ova protein (~25–34%) with the C-Ova immunogenic peptide (~22–40%). These data indicated that MHC II loading of immunogenic peptides and/or the translocation of MHC II-immunogenic peptide complexes to the cell surface may also contribute to the reduction in macrophage AP following Exh, along with reduced antigenic peptide generation. This may seem at odds with our previous finding that the total amount of MHC II is not different among the groups (5). However, total levels of MHC II on the surface of the macrophage as measured by flow cytometry are, at best, only a gross estimation of AP ability. The best indicator would be the number of MHC II molecules that specifically had C-Ova in their cleft. Unfortunately, this is very difficult to measure.

An increase in phagolysosomal pH may explain reduced macrophage AP, antigenic peptide generation, and loading onto MHC II, processes that are all pH dependent. That exercise or stress might affect phagolysosomal pH in macrophages is supported by the demonstration of increased lysosomal pH in liver cells following exhaustive exercise (27) and increased cellular pH in TG-elicited peritoneal macrophages following exposure to oxidative (H$_2$O$_2$) stress (4). Increases in pH would reduce the ability of macrophages to generate antigenic peptides (lysosomal enzymes and proteases are pH dependent) and to load those immunogenic peptides onto MHC II molecules (13). Indeed, drugs (i.e., chloroquine, NH$_4$Cl) that raise the pH of intracellular vesicles inhibit macrophage AP by inhibition of acid proteases (cathepsins B, D, and L), which in turn inhibit antigen processing (34). It is known that cathepsin D is of vital importance in macrophage AP of C-Ova to antigen-specific T cells (25). Therefore, the underlying mechanism responsible for the depressed macrophage AP following Exh may be an increase in phagolysosomal pH, which inhibits antigen processing, MHC II loading, and the translocation of MHC II-immunogenic peptides to the cell surface. This contention awaits definitive experimentation.

Most evidence available indicates that acute or short-term (4–7 days) exercise or exercise training enhances many functions of peritoneal macrophages, including tumor killing (17, 32, 33), NO production (17), chemotaxis toward antigenic stimuli (11, 21), phagocytosis of...
opsonized Candida albicans (7, 10, 18, 21), metabolic and lysosomal enzyme activity (10), and microbicidal activity as measured by nitroblue tetrazolium reduction (8). The mechanism(s) responsible for these enhancements in macrophage function remains to be elucidated. In contrast, exhaustive exercise has been found to suppress intrinsic alveolar macrophage antiviral activity through a β-adrenergic receptor-mediated mechanism (16). The role of stress hormones in the mediation of the exercise-induced suppression in peritoneal macrophage AP awaits further study. On the basis of the available observations regarding exercise and macrophage function, it appears that exercise activates some effector macrophage functions, whereas other functions such as AP are suppressed. Although speculative, this may lead to enhanced innate immune function but suppressed lymphocyte-mediated immunity.

In conclusion, the suppression in peritoneal macrophage AP in response to Exh is due to an intracellular defect (most probably a combination of reduced antigenic breakdown, MHC II loading, and MHC II-C-Ova peptide complex surface expression) in the macrophage antigen processing pathway. Exercise stress-induced suppression of macrophage AP is not related to the production of autocrine or paracrine factors by macrophages. The impairment in macrophage AP as a result of diminished intracellular processing may help to explain some of the previously reported immune suppression following repeated exhaustive exercise (19).

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