Toll-like receptor 4 and CD14 mRNA expression are lower in resistive exercise-trained elderly women

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Submitted 10 April 2003; accepted in final form 23 June 2003

Flynn, Michael G., Brian K. McFarlin, Melody D. Phillips, Laura K. Stewart, and Kyle L. Timmerman. Toll-like receptor 4 and CD14 mRNA expression are lower in resistive exercise-trained elderly women. J Appl Physiol 95: 1833–1842, 2003.—The purpose of this study was to examine the influence of resistive exercise training and hormone status on mRNA expression of toll-like receptor 4 (TLR4), CD14, IL-1β, IL-6, and TNF-α. Resistive exercise-trained women on “traditional” hormone replacements [hormone replacement therapy (HRT), n = 9], not taking hormones (NHR, n = 6), or taking medications known to influence bone (MIB, n = 7) were compared with untrained subjects not taking supplemental hormones (Con, n = 6). Blood was taken from trained subjects before, immediately after, and 2 h after resistive exercise (same time points for resting Con). TLR4 mRNA expression (RT-PCR) was not different among groups or across time but was significantly (P = 0.044) lower (1.9-fold) when trained groups were collapsed and compared with Con. There was also a significant group effect (P < 0.0001) for TLR4 mRNA when expressed per monocyte. CD14 expression was significantly (P = 0.006) lower (2.3-fold) for training groups collapsed and compared with Con. CD14 mRNA, expressed per monocyte, was significantly lower immediately after resistive exercise for NHR, HRT, and MIB compared with Con. Few significant effects detected for IL-6, IL-1β, and TNF-α mRNA, but there was a significant group effect (P < 0.0001) for TNF-α mRNA expressed per monocyte (Con > HRT, NHR, MIB). These findings suggest that there may be a resistive exercise training-induced reduction in TLR4/CD14 expression in older women. Further research is needed to determine whether lower TLR4/CD14 could explain the lower LPS-stimulated inflammatory cytokines observed in these women. Toll receptors are transmembrane proteins, originally discovered in Drosophila, that are highly conserved between insects and humans (18, 20). In Drosophila, toll receptors are associated with ontogenesis and antifungal defense (20). Human homologs of the toll receptors have been identified and are called toll-like receptors (TLR). There are 10 known TLR, and all of those with well-characterized functions appear to be responsible for recognizing pathogen-associated molecular patterns (1, 5). For example, TLR2 recognizes peptidoglycan and lipoproteins commonly found on gram-positive bacterial cell walls, and TLR3 has recently been found to recognize double-stranded RNA (2). TLR4, working in conjunction with the LPS receptor CD14, is the primary signaling receptor for gram-negative bacterial LPS (5). LPS is commonly used as a mitogen in mononuclear cell (MNC) or whole blood stimulation assays (9, 24, 28, 32) and initiates intracellular signaling events via CD14 and TLR4. LPS-stimulated inflammatory cytokine production has been reported to be lower in younger, compared with older, subjects (11, 28, 29), lower in pre- vs. postmenopausal women (8, 26), and lower in trained than untrained subjects (25, 31).

Based on previous research, we hypothesize that lower TLR4 expression could help to explain both the blunted response to LPS after acute exercise (16) and reduced LPS-stimulated cytokine production in trained compared with untrained subjects. Therefore, reduced mitogen-stimulated TNF-α, IL-1α, and IFN-γ production in older subjects (31), and LPS-stimulated IL-6 production was reported to be blunted in young, highly trained athletes compared with recreationally active controls (25). Evidence of reduced mitogen-stimulated inflammatory cytokine production with exercise training (31) or in highly trained subjects (25) led us to examine potential mechanisms for these apparent exercise training-induced changes. In addition, hormone status has been reported to influence mitogen-stimulated cytokine production and serum levels of these cytokines (6, 23), and we further sought to determine whether hormone status influenced our primary dependent variables.

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the main purpose of this study was to measure TLR4, CD14, IL-6, IL-1β, and TNF-α mRNA expression in blood samples obtained from resistive exercise-trained and untrained women and compare these findings with LPS-stimulated inflammatory cytokine production measured in the same subjects.

Several researchers reported that hormone status could influence mitogen-stimulated IL-6, IL-1β, and TNF-α production as well as serum and plasma levels of these proteins (6, 23). Bismar et al. (6) found that bone cells obtained from subjects who had discontinued estrogen replacement produced significantly more IL-1, TNF-α, and IL-6 than those of premenopausal women. In addition, Pacifi et al. (23) found monocyte production of IL-1β to be higher in untreated postmenopausal than premenopausal or hormone replacement therapy (HRT)-treated (estrogen and progesterone) postmenopausal women. In a preliminary study, our laboratory (32) also found significantly higher LPS-stimulated IL-6 production in postmenopausal women who were not taking hormones (NHR) compared with that in premenopausal women. As expected, estradiol levels were significantly lower in the postmenopausal subjects, and we speculated that estradiol might have been at least partially responsible for the differences. Therefore, a second purpose of this investigation was to determine whether hormone replacement, taken as “traditional” conjugated estrogen-progesterone, would influence the mRNA transcripts for TLR4, CD14, IL-6, IL-1β, and TNF-α.

METHODS

Subjects

The subjects in this study were apparently healthy women between the ages of 65 and 85 yr (Table 1). All subjects read and signed informed consent (Purdue University Committee on the Use of Human Research Subjects no. 99-024) before participating in the study. Subjects were assigned to groups based on their training status (resistive trained or sedentary), and trained women were further subdivided based on hormone status: 1) women taking “traditional” HRT (n = 9), 2) NHR women (n = 6), and 3) women not taking hormones but taking other medications known to influence bone (MIB; n = 7), such as selective estrogen receptor modulators (e.g., Evista) and bisphosphonates (e.g., Fosamax). Women in the same age range and with similar health status, who were not participating in regular exercise and were not taking supplemental hormones, were used as controls (Con, n = 6). These women were selected at the conclusion of a weight-training study, which employed the screening, exclusionary criteria, training, and posttraining “standardized exercise test” described below. The impetus to study TLR4 came after the start of training, which explains the present study design.

Screening and Preliminary Testing

All subjects underwent thorough medical screening before being enrolled in the study. Initial screening involved a preliminary medical and exercise history and approval from the subjects’ personal physicians. Subjects with chronic and debilitating arthritis, central or peripheral nervous system disorders, previous stroke, acute or chronic infection, major affective disorder, or human immunodeficiency virus infection and autoimmune diseases or metabolic disorders (Type 1 diabetes mellitus) were excluded from participation, as were subjects who were smokers or smokeless tobacco users, oral corticosteroid users, bedridden within 3 mo of the study, or had surgery within the previous 3 mo.

The initial screening was followed by a medical history and physical exam by the study physician, resting ECG and blood pressure, a submaximal treadmill stress test (85% of maximal heart rate), and resistive exercise stress test, which consisted of ECG and blood pressure monitoring during one repetition maximum (1-RM) and eight repetitions maximum (8-RM) leg press exercise. Subjects also completed a “get-up-and-go test” to screen for difficulties with ambulation, balance, or lower extremity problems. Additionally, the study physician performed a musculoskeletal screen to exclude patients who might have difficulty with the exercise training.

Acclimation and Resistive Exercise Training

During an acclimation week, all subjects, including Con, were taught the correct procedure for performing the following exercises: seated leg press, knee extension, knee flexion, upright chest press, chest “flys,” lat pull-down, shoulder press, upright rows, hip adduction, and leg abduction (Cybex Resistance Training, Ottowa, MN). Strength testing was also completed during the acclimation week, such that each subject’s 8 RM was determined for each of the exercises on the first day of the acclimation week. On the second day, subjects performed three sets of each exercise at 50% of their estimated 1 RM. Finally, on the third day of the acclimation week, the 8 RM was determined a second time for each subject, and the 1 RM was determined for chest press, leg extension, and leg curl. Subjects were allowed at least a 24-h recovery between acclimation sessions.

After acclimation, HRT, NHR, and MIB subjects trained three times per week, on alternate days, performing three

Table 1. Descriptive data for the subjects in the trained groups taking “traditional” hormone replacements, not on hormone replacements, those taking other medications known to influence bone, and for untrained controls

<table>
<thead>
<tr>
<th></th>
<th>HRT</th>
<th>MIB</th>
<th>NHR</th>
<th>Con</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>74.02 ± 5.62</td>
<td>72.01 ± 7.46</td>
<td>71.60 ± 6.47</td>
<td>74.20 ± 6.98</td>
</tr>
<tr>
<td>Height, cm</td>
<td>161.28 ± 5.83</td>
<td>162.0 ± 3.67</td>
<td>161.33 ± 7.54</td>
<td>161.55 ± 3.71</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>69.17 ± 10.80</td>
<td>66.52 ± 12.46</td>
<td>66.83 ± 11.30</td>
<td>68.7 ± 7.34</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>28.67 ± 4.53</td>
<td>28.05 ± 5.24</td>
<td>26.04 ± 6.14</td>
<td>26.68 ± 6.14</td>
</tr>
<tr>
<td>%Body fat</td>
<td>31.37 ± 5.24</td>
<td>31.70 ± 5.39</td>
<td>32.38 ± 4.50</td>
<td>32.81 ± 2.59</td>
</tr>
<tr>
<td>8-RM chest press, kg</td>
<td>23.69 ± 5.96</td>
<td>27.59 ± 8.47</td>
<td>24.17 ± 6.05</td>
<td>13.63 ± 9.09*</td>
</tr>
<tr>
<td>8-RM leg press, kg</td>
<td>91.1 ± 16.62</td>
<td>94.29 ± 26.45</td>
<td>98.34 ± 15.39</td>
<td>65.84 ± 14.89*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of subjects. HRT, hormone replacement therapy; NHR, not on hormone replacements; MIB, medications known to influence bone; Con, untrained controls; 8 RM, eight repetitions maximum. *Significantly different from trained groups, P < 0.05.
sets of the same 10 upper- and lower-body exercises, whereas the Con returned to their normal activities. During the first week of training, the subjects performed eight repetitions in the first two sets at a resistance equal to 0% of the 1 RM and in the third set performed as many repetitions as possible. At least 1-min recovery was required between sets. Intensity was increased to 80% of 1 RM for the second week. When a subject was able to complete more than 12 repetitions in the third set, the resistance was increased for the following session. In addition, the 8 RM for each exercise and the 1 RM for chest press, leg extension, and leg curl were restated during the fifth week of training, and the intensity of training was adjusted accordingly. The 8 RM and 1 RM were also determined at the end of training (week 10).

**Experimental Trial**

One week after their last training session, subjects reported to the laboratory between 0630 and 0730 after an overnight fast and rested quietly in a chair for 15 min, at which point a venous blood sample (20 ml) was taken from a prominent arm vein in the antecubital region (Pre). After a 10-min warm-up on a treadmill, subjects in the HRT, NHR, and MIB groups performed three sets of 10 resistive exercises at an intensity calculated to elicit 80% of 1 RM, with at least 1.5-min recovery between sets. Eight repetitions were performed in the first two sets, and the last set was performed to muscular failure. Additional blood samples were taken immediately after (Post) and 2 h after resistive exercise (2H) and at the same time points from the Con group, who sat quietly in the laboratory during the exercise sessions. Venous blood samples were collected into evacuated tubes containing K3-EDTA (ethylene-diaminetetraacetic acid), and aliquots were immediately frozen (−2 min) at −80°C until total RNA extraction.

**Blood Analysis**

**Total cellular RNA extraction.** Total cellular RNA was extracted from previously frozen EDTA-treated whole blood aliquots (200 µl) by using a standard phenol-chloroform extraction method (TRI-Reagent BD; Sigma-Aldrich, St. Louis, MO). Purified total cellular RNA was analyzed for integrity of the 18S and 28S ribosomal bands on a 1% agarose-Tris-acetic acid-EDTA gel (VWR Scientific) for 2 h at 60°C.

**RT-PCR of total cellular RNA.** After confirmation of total RNA integrity, each RNA sample was diluted to a concentration of 200 ng/µl and reverse transcribed into complementary DNA by using a commercially available kit (RT System; Promega Life Science). The mRNA expression was analyzed by using a semiquantitative PCR technique (Promega). PCR was optimized for each primer pair (Table 2) and completed utilizing mRNA-specific primers for TLR4 (30 cycles), CD14 (30 cycles), IL-6 (30 cycles), IL-1β (30 cycles), and TNF-α (30 cycles) (Ransom Hill Biosciences, Ramona, CA). Primers were also included for the gene that encodes GAPDH to control for between-sample variations in loaded quantities of complementary DNA. PCR products were analyzed on a 2% agarose/Tris-acetic acid-EDTA gel (VWR Scientific) for 1 h at 110 V. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed (Polaroid DS-34 camera) (Fig. 1). PCR band intensity was determined by using Scion Image Analysis Software (National Institutes of Health) and normalized against GAPDH. mRNA values were expressed normalized against GAPDH and also "corrected" for the number of monocytes, leukocytes, and MNCs in the blood sample.

**Cytokine stimulation assays.** Cytokine production was assayed by using a modification of whole blood methods (9, 37), by culturing whole blood (1:10 with RPMI supplemented with 2 mM L-glutamine and penicillin/streptomycin) in 2.0-ml, 24-well flat-bottomed plates (Corning). Cultures were stimulated with the polyclonal activator LPS (from Salmonella enteriditis; 25 µg/ml final concentration; Sigma-Aldrich). All blood samples were diluted, cultured, and placed into the incubator within 2 h of blood sampling. After a 24-h incubation (37°C, 5% CO2, humidified environment), supernatants were harvested, centrifuged (500g for 10 min) to eliminate cellular debris, and stored at −80°C until analyzed for IL-1β, IL-6, and TNF-α by using an ELISA.

**Cytokine analysis.** The IL-1β, IL-6, and TNF-α protein concentration in supernatant from LPS-stimulated cultures was analyzed by using separate ELISA kits (BD PharMingen). After incubation of samples and standards in wells of a previously coated (anti-cytokine monoclonal antibody) 96-well plate (Nunc Maxisorb), a working detector (polyclonal anti-cytokine antibody and avidin-horseradish peroxidase conjugate) was used to determine the quantity of cytokine present. A substrate was added, and optical density was read at 450 nm with a 630-nm reference (BioTek Elx800, Winooski, VT). Cytokine stimulation data were expressed as concentration in the culture supernatant and per monocyte in culture.

**Cell counts and differential.** Total leukocyte count was determined after erythrocyte lysis on a Coulter Z2 particle counter (Beckman-Coulter, Miami, FL). Manual differentials were performed on Wright-Giemsa-stained whole blood smears.
Table 2. mRNA PCR primer sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
<th>Sequence (3' → 5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toll-like receptor 4</td>
<td>Left</td>
<td>tga gca gtc gtc gtg gta tc</td>
</tr>
<tr>
<td>CD14</td>
<td>Right</td>
<td>gag ggc ttc ttt ctc gag gtc tc</td>
</tr>
<tr>
<td>IL-6</td>
<td>Left</td>
<td>gca gas aac ggt ggg tgt gt</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Right</td>
<td>gca gaa gag agc cca cca ac</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Left</td>
<td>aag sat tca aac tgg ggc ct</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Right</td>
<td>gca gaa ggc ctc agc tgc ac</td>
</tr>
</tbody>
</table>

Statistical Analyses

Statistical analyses were performed by using a personal computer-based statistics program (SPSS version 10.1). Messenger RNA expression was analyzed by using a 4 × 3 factor ANOVA with repeated measures on the second factor. The first factor had four levels of group (HRT, NHR, MIB, and Con), and the second factor had three levels of time (Pre, Post, and 2HPO). Data were checked and cleared for assumptions of normality (Shapiro-Wilk test) and constancy of variance (residual plotting) before analysis by ANOVA. A secondary analysis was conducted with all trained subjects pooled and compared with Con subjects by using a 2 × 3 factor ANOVA with repeated measures on the second factor. Subjects were also divided into two groups, irrespective of training status, based on their pre-TLR4 mRNA expression. The pre-LPS-stimulated IL-6, IL-1β, and TNF-α production of these groups of “high TLR4 expressers” and “low TLR4 expressers” was then compared by using one-way ANOVA.

Significant effects (P < 0.05) were further analyzed to determine location of significance by using a Student-Newman-Keuls post hoc test. Correlation analyses were performed by using a Pearson stepwise correlation matrix. Correlations were calculated between mRNA for TLR4 and CD14 (total and per monocyte) and LPS-stimulated cytokine production (total and per monocyte).

Table 3. Cell counts before and immediately and 2 h after resistive exercise for trained subjects taking HRT, NHR, MIB, and for Con

<table>
<thead>
<tr>
<th></th>
<th>HRT</th>
<th>MIB</th>
<th>NHR</th>
<th>Con</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Leukocyte, × 10⁹/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>4.93 ± 0.44</td>
<td>4.97 ± 0.50</td>
<td>4.87 ± 0.54</td>
<td>5.31 ± 0.54</td>
</tr>
<tr>
<td>Post</td>
<td>7.41 ± 0.56†</td>
<td>6.32 ± 0.64†</td>
<td>6.23 ± 0.69†</td>
<td>4.78 ± 0.69</td>
</tr>
<tr>
<td>2HPO</td>
<td>6.30 ± 0.40</td>
<td>5.70 ± 0.46</td>
<td>5.94 ± 0.49</td>
<td>5.59 ± 0.49</td>
</tr>
<tr>
<td>Mononuclear, × 10⁹/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>1.98 ± 0.26</td>
<td>1.59 ± 0.29</td>
<td>2.26 ± 0.32</td>
<td>2.28 ± 0.32</td>
</tr>
<tr>
<td>Post</td>
<td>2.85 ± 0.30†</td>
<td>1.98 ± 0.34</td>
<td>2.62 ± 0.36†</td>
<td>1.62 ± 0.36</td>
</tr>
<tr>
<td>2HPO</td>
<td>2.15 ± 0.23</td>
<td>1.68 ± 0.26§</td>
<td>2.39 ± 0.28</td>
<td>1.95 ± 0.28</td>
</tr>
<tr>
<td>Monocyte, ×10⁹/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>3.30 ± 0.60</td>
<td>4.60 ± 0.33</td>
<td>4.30 ± 1.19</td>
<td>3.10 ± 0.90</td>
</tr>
<tr>
<td>Post</td>
<td>4.60 ± 0.94*</td>
<td>7.00 ± 0.92*</td>
<td>7.30 ± 1.28*</td>
<td>2.20 ± 0.60*</td>
</tr>
<tr>
<td>2HPO</td>
<td>2.70 ± 0.84</td>
<td>4.10 ± 0.54</td>
<td>5.30 ± 1.69</td>
<td>2.80 ± 0.55</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. Pre, before exercise training; Post, immediately after exercise training; 2HPO, 2 h after exercise training. *Significant (P < 0.05) difference from Pre. †Significant (P < 0.05) difference from Con-Post. §Significant (P < 0.05) difference from Con-Pre. Statistical Analyses

RESULTS

Subjects

There were no significant differences among groups for age, height, weight, body mass index, or any other descriptive measures (Table 1). Results from the 8-RM tests show that there were significant strength differences between trained (HRT, NHR, MIB) and untrained subjects (Con) (Table 1).

White Blood Cell Counts

ANOVA revealed a significant interaction effect for the total leukocyte number (P < 0.0001) in whole blood, and the post hoc test determined that the Post values were higher for the trained groups compared with Con (Table 3). MNC number was also higher (P = 0.002) Post for trained groups compared with Con and the 2HPO value for NHR was different from MIB at 2HPO (Table 3). Monocyte number was significantly different across time (P < 0.002), such that the Post sample was significantly higher than the Pre and 2HPO (Table 3). No significant group (P = 0.051) or interaction effects were found for monocytes.

LPS Receptor TLR4 and CD14 mRNA Expression

When mRNA for TLR4 was expressed normalized against GAPDH, there were no significant (P > 0.05) fold differences for time, group, or interaction effects (Fig. 2A). The group effect did reach the level of statistical significance (P = 0.044) when trained subjects were pooled and compared with Con, such that the TLR4 mRNA group mean for trained subjects (collapsed across time) was 1.9-fold lower than that of Con. When TLR4 mRNA was normalized against GAPDH and expressed per monocyte, there was a significant group effect (P < 0.0001), such that Con had 2.3-, 4.5-, and 3.0-fold greater mRNA expression than HRT, NHR, and MIB, respectively (Fig. 2B). When TLR4 was normalized against GAPDH and expressed per leukocyte, there was a significant group effect (P = 0.044),...
but the post hoc revealed that only NHR was significantly lower (2.7-fold) than Con. There were no significant group, time, or interaction effects when TLR4 was expressed per MNC (data not shown). There were also no significant effects observed for IL-6 mRNA when the training groups were pooled (normalized against GAPDH) and compared with Con.

There were no significant time, group, or interaction effects for IL-1β mRNA expression normalized against GAPDH (Fig. 5A) per monocyte (Fig. 5B; P = 0.05 for group effect), leukocyte, MNC (P = 0.056 for group), or when the training groups were pooled and compared with Con.

There were no significant time, group, or interaction effects for TNF-α mRNA expression normalized against GAPDH (Fig. 6A), even when training groups were pooled and compared with Con. There were no significant time or interaction effects for TNF-α mRNA expression per monocyte (Fig. 6B), but there was a significant group effect (P < 0.0001). In this case, the TNF-α expression for Con was higher than that for HRT (2.2-fold), NHR (1.6-fold), and MIB (2.7-fold). There were no significant group, time, or interaction effects when TNF-α mRNA was expressed per leukocyte, and no group or interaction effects when expressed per MNC, but there was a significant time effect (P = 0.035), such that the grand mean for the Post value was lower than that for the Pre value (data not shown).

**IL-6, IL-1β, and TNF-α mRNA Expression**

There were no significant time, group, or interaction effects for IL-6 mRNA expression normalized against GAPDH (Fig. 4A) or IL-6 mRNA expression per monocyte (Fig. 4B), per MNC, or per leukocyte (data not shown). There were also no significant effects observed for IL-6 mRNA when the training groups were pooled (normalized against GAPDH) and compared with Con.
The cytokine stimulation data were included in the present study for correlation with TLR4 and CD14 expression and to compare inflammatory cytokine production in groups of high and low TLR4 expressers. However, it is important to note that there were significant group effects for LPS-stimulated cytokine production, expressed per monocyte in culture, such that the trained groups had significantly lower IL-6 ($P = 0.031$), IL-1β ($P = 0.037$), and TNF-α ($P = 0.046$) compared to Con. Correlations between mRNA expression for TLR4 normalized vs. GAPDH and LPS-stimulated cytokine production (total) revealed weak, largely negative relationships ($P > 0.05$) (data not shown).

**TLR4 Expression and LPS-stimulated Inflammatory Cytokine Production**

As described in Statistical Analyses, subjects were divided, irrespective of training status or hormone group, into groups based solely on resting (Pre) TLR4 mRNA (per monocyte) and CD14 mRNA. These groups of “high TLR4 expressers” ($n = 14$) and “low TLR4 expressers” ($n = 14$) were compared for LPS-stimulated IL-1β, IL-6, and TNF-α. ANOVA revealed significantly lower IL-6 ($P = 0.031$), IL-1β ($P = 0.037$), and TNF-α ($P = 0.046$) protein concentration in the supernatant for the low vs. the high TLR4 expressers. IL-6 levels were $277.8 \pm 35.1$ and $176.6 \pm 33.2$ fg/monocyte for high and low, and Post TLR4 mRNA were not consistently correlated with IL-6, IL-1β, and TNF-α production, but there was a significant relationship between CD14 Pre mRNA and IL-1β production ($r = 0.394$) for the 2HPO sample (Table 4). Not surprisingly, there were consistent, significant relationships between cytokines measured at the same time point, from the same stimulation wells (e.g., Pre IL-6 vs. Pre TNF-α, $r = 0.083$). There were also consistent, significant relationships between inflammatory cytokine production at different time points (e.g., Pre TNF-α and Post IL-6, $r = 0.725$). Correlations between mRNA expression for TLR4 normalized vs. GAPDH and LPS-stimulated cytokine production (total) revealed weak, largely negative relationships ($P > 0.05$) (data not shown).
respectively. IL-1β levels were 101.6 ± 14.6 and 46.0 ± 8.7, and TNF-α levels were 22.4 ± 3.4 and 10.1 ± 2.2 fg/monocyte for high and low groups, respectively.

**DISCUSSION**

TLR are important mediators of both the innate and cytokine-activated, cell-mediated immune systems. TLR4, working in conjunction with CD14, is the primary signaling receptor for bacterial gram-negative LPS, leading to monocyte activation and, among other things, increased inflammatory cytokine production (36). LPS has been used as a mitogen in several studies in which differences in inflammatory cytokine production between younger and older subjects was observed (28, 29). Additionally, exercise training was shown to reduce mitogen-stimulated TNF-α and IL-1 production (31), and LPS-stimulated IL-6 was reported to be lower in trained compared with Con subjects (25). Finally, estrogen status has also been proposed to influence inflammatory cytokine production (6, 23). Thus the influence of both hormone and training status on TLR4/CD14 mRNA expression was examined in this investigation.

Wang et al. (35) found that cell-surface expression of TLR4 (measured using flow cytometry) on human gingival fibroblasts was associated with higher IL-6 and IL-1 production by these cells. We ran correlations on the data of Wang et al., which revealed strong relationships between mean fluorescence intensity for TLR4 and LPS-stimulated IL-6 (r = 0.89) and IL-1 production (r = 0.67). These and other findings led us to investigate TLR4 as a possible mediator of the training-induced reductions in LPS-stimulated cytokine production observed in the present study. To our knowledge, there are no published data comparing TLR4 expression in trained and untrained individuals. Thus we believe that these data provide novel information in an area of research with potential to provide insight into the nature of the relationship between inflammatory cytokines and exercise training, “typical aging,” and onset of “age-related” diseases.

The subjects in the present study were divided into two groups: one with the highest (n = 14) and the other with the lowest (n = 14) Pre TLR4 mRNA levels. There are potential limitations comparing TLR4 mRNA expression from unstimulated blood samples with cytokine-activated, cell-mediated immune systems.
kine production after LPS stimulation, and we acknowledge that mRNA for TLR4 cannot be directly linked to cytokine production. Nevertheless, as in the study by Wang et al. (35) using cell-surface TLR4 expression, there were significant differences between the groups of high and low TLR4 expressers for LPS-stimulated IL-6, IL-1β, and TNF-α concentrations. With respect to training classification, three of the four highest TLR4 mRNA expressers were Con subjects, and all six Con subjects were in the high-expressor group. There were also strong correlations between mRNA TLR4 2HPO and per-monocyte LPS-stimulated IL-6, IL-1β, and TNF-α production at the same time points and similar relationships between 2HPO CD14 mRNA and 2HPO LPS-stimulated cytokine production. There were, however, few significant relationships between TLR4 or CD14 mRNA and LPS-stimulated inflammatory cytokines at either Pre or Post time points, and we are unable to explain this discrepancy. Cell-surface expression of TLR4/CD14 (flow cytometry) may provide a better indication of a cell’s responsiveness to LPS, and we are examining this in ongoing studies. Preliminary results show that cell-surface expression of TLR4 by CD14+ cells (monocytes and dendritic cells) is lower in trained than in untrained elderly women (M. G. Flynn, unpublished observation).

Unstimulated whole blood samples were used for RNA isolation and subsequent RT-PCR analysis in the present study. These whole blood samples were obtained at Pre, Post, and 2HPO or at the same time points for resting Con. The Con provided a safeguard against circadian variation, but, in retrospect, it would help to also have exercise data on the Con. Nevertheless, among the many comparisons for mRNA, there was only one significant time effect (TNF-α Post grand mean was lower than Pre). Thus it appears that the acute resistive exercise stimulus did not substantially alter mRNA expression in whole blood, and, with respect to TLR4/CD14 mRNA, the most “potent” independent variable appeared to be training status. The cross-sectional nature of the study design, however, does not exclude the possibility that differences in TLR4/CD14 mRNA expression between groups existed at the start of the study.

mRNA was normalized vs. GAPDH and also expressed per leukocyte, per MNC, and per monocyte. Considering the present study design, we believe that it was important to consider shifts in white blood cells likely to occur during exercise that could influence the RT-PCR analyses. mRNA analyses were made on whole blood for comparison with whole blood LPS-stimulation assays. MNC or monocyte isolations could have been performed, but the extensive processing required during isolation could influence gene expression (4).

LPS stimulation requires the presence of the LPS receptor (CD14) and TLR4, which suggests that monocytes and other CD14+ cells (e.g., myeloid dendritic cells) in our cultures would be most responsive to LPS. However, because TLR4 is reported to be strongly expressed in cells of myeloid origin (27), we also expressed mRNA values per leukocyte and per MNC to further control for the fact that all measured mRNA did not come from monocytes. Correcting for shifts in leukocyte subsets resulted in statistical differences that were not detected when mRNA was normalized vs. GAPDH. For example, expressing mRNA per monocyte resulted in significantly lower TLR4 and CD14 in trained groups compared with Con, but these differences were likely influenced by exercise-induced monocyte shifts. However, there were significant differences for mRNA normalized against GAPDH when the trained groups were collapsed and compared with Con. In addition, post hoc power analysis suggests that individual group differences for TLR4 and CD14 mRNA, normalized against GAPDH, would have been significant if subject number had been increased by only two subjects per group. Thus it appears that 10 wk of resistive exercise training influenced mRNA gene expression for the receptors that transduce the LPS signal.

The only apparent difference between the NHR and Con groups was resistive exercise status, but there was a significantly lower TLR4 and CD14 mRNA expression for NHR (trained) than Con. What made these findings most surprising was that the changes occurred in circulating blood cells, suggesting that muscle contraction during resistive exercise training influenced these cells. There are a host of potential candidates that could mediate contraction-induced changes in circulating monocytes, including endocrine mediators or a substance released by contracting skeletal muscle.

Heat shock proteins 60 (HSP60) and 72 (HSP72) have been shown to stimulate monocytes in an LPS-like manner (3, 33), and HSPs (e.g., 60) bind to and activate TLR2 and TLR4 to stimulate inflammatory cytokine production from monocytes and dendritic cells, thus exhibiting “chaperokeine” effects (3). Moderate-intensity exercise has been found to increase muscle HSP (HSP60 and HSP72) (19), and Walsh et al. (34) reported that HSP72 was released into the circulation after a submaximal run of 60 min. In a later paper from the same group, the authors speculated that eccentric contractions might be required to elicit the release of HSP (13), but they have also found that the liver may be a major source of serum HSP72 increases during exercise (12). The resistive exercise training completed by our subjects involved eccentric contraction, the forced lengthening of skeletal muscle that can lead to muscle damage, which might be expected to induce a release of HSP into circulation. Therefore, it is possible that HSPs, released into the circulation during or after resistance exercises, could provide a means for influencing either the expression of TLR4 or the LPS-stimulated production of IL-6, IL-1β, or TNF-α in monocytes.

There are also several other intriguing possibilities to explain a training-induced downregulation of TLR4/CD14, among which are increased stimulation of the IL-1 receptor during and after repeated bouts of exercise or “cross talk” among TLR receptors (10). Because the IL-1 receptor and TLR4 have similar
cytoplasmic domains, what is commonly called the toll/IL-1R homologous region (TIR domain), it is possible that exercise-induced elevations in IL-1β could influence TLR4 expression, function, or signaling in a fashion similar to the endotoxin tolerance that can be elicited by LPS (10).

Ishizashi et al. (16) reported that a single bout of exercise induced inhibition of endotoxin shock (i.e., increased survival rate of LPS exposed mice). These authors speculated that the effect was a result of decreased LPS-induced production of IL-1 and IL-6 in these animals. Thus it is possible that even a single bout of exercise could provide “endotoxin tolerance,” but we do not know if chronic training exerts its effect in a similar fashion. Except for the presence of a significant interaction for Post CD14 mRNA (Fig. 2B), there was little evidence of an acute exercise effect on mRNA expression up to 2 h postexercise in our trained subjects.

In our preliminary experiments, LPS-stimulated IL-6 production was greater in older (65–80 yr) than younger women (18–30 yr) at rest and also greater during an exercise trial (32). As expected, estradiol was substantially lower in the postmenopausal women (NHR); therefore, we reasoned that hormone status might explain at least part of the difference between women in different age groups. Estradiol was roughly fourfold higher (data presented elsewhere) in HRT compared with NHR and MIB in the present study, but there were no significant differences between trained groups for LPS-stimulated inflammatory cytokine production or TLR4 and CD14 mRNA expression. Therefore, we were unable to measure a difference in response between trained women of different hormone status; however, it is possible that the training obfuscated the effect.

Other researchers have observed exercise training-induced differences in inflammatory cytokine production (14, 24, 31). Pool et al. (25) reported that LPS-stimulated IL-6 production was significantly lower in triathletes and professional rugby players compared with recreationally trained controls, but the athletes had higher unstimulated IL-6 production than controls, and the “deficit” in IL-6 production was observed when changes in IL-6 were measured after LPS stimulation. Smith et al. (31) found that 6 mo of combined endurance and resistive exercise training substantially reduced IFN-γ, IL-1α, and TNF-α using a different mitogen, phytohemaglutinin, that has not been identified as a ligand for TLR. These findings by Smith et al. could suggest, among several possibilities, that either TLR4 may not be responsible for these changes; that exercise-training influences “downstream” cytokine production pathways; or exercise training influences a broad range of cellular receptors, including those that transduce the phytohemaglutinin signal.

In summary, resistive exercise training appeared to lower TLR4 and CD14 mRNA expression in older women, regardless of hormone status. In addition, LPS-stimulated IL-6 and TNF-α production was lower in resistive exercise-trained women compared with Con, and IL-6, IL-1β, and TNF-α production was statistically greater in the 14 highest, compared with the 14 lowest, TLR4 “expressers.” These data lead us to suggest that resistive exercise training could influence receptor-driven cytokine signaling by endotoxin.

The authors thank the subjects for their effort and dedication, which contributed to the success of this research.

DISCLOSURES

This investigation was funded, in part, by a Pilot Research Grant from the Purdue University Gerontology Program.

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