Physical Activity Status, But Not Age, Influences Inflammatory Biomarkers and Toll-Like Receptor 4

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Background. Chronic inflammation has been implicated in the development of cardiovascular disease, diabetes mellitus, cachexia, and osteoporosis. Regular physical activity has been purported to possess “anti-inflammatory” properties which may limit chronic inflammation. Recently, we hypothesized that toll-like receptor 4 (TLR4) may play a role in activity-induced modulation of inflammation. Therefore, the purpose of this study was to determine the association between age, physical activity status, biomarkers of inflammation, and TLR4.

Methods. Male and female participants (n = 84) were recruited to fill one of the following groups: young (18–30 years), active; young, inactive; old (60–80 years), active; or old, inactive. To assess physical activity status, participants completed a Paffenbarger Physical Activity Questionnaire and a modified Balke submaximal treadmill test. After grouping and screening, participants were given a standard mixed diet to consume 24 hours prior to arriving at the laboratory. Participants were instructed to consume all food by 10 PM the night prior to blood sampling (8-hour fast). Following 30 minutes of seated rest in a quiet room, venous blood samples were collected. Lipopolysaccharide-stimulated inflammatory cytokine production and plasma high-sensitivity C-reactive protein (hsCRP) were determined by enzyme-linked immunosorbent assay, and TLR4 expression was determined by flow cytometry.

Results. Lipopolysaccharide-stimulated interleukin-6, interleukin-1β, and tumor necrosis factor-α production, TLR4 expression, and hsCRP were significantly lower in active compared to inactive participants (p < .05). Also, older participants had significantly higher hsCRP than young participants had (p < .05).

Conclusions. The findings of the present study support previous reports which infer that acute exercise or a physically active lifestyle may possess anti-inflammatory properties. Also this study, along with previous work from our laboratory, suggests that TLR4 may play a role in regulating the link between inflammatory cytokine production and a physically active lifestyle.

Chronic inflammation has been implicated in the development of osteoporosis, diabetes mellitus, cachexia, and cardiovascular disease (1,2). A physically active lifestyle has been reported to exert anti-inflammatory effects, with minimal side effects (3–6).

Chronic inflammation can be assessed by measurement of various inflammatory biomarkers, including inflammatory cytokines (interleukin [IL]-6, IL-1β, and tumor necrosis factor [TNF]-α) and acute phase proteins (high-sensitivity C-reactive protein [hsCRP]) (7,8). The maximal capacity of peripheral blood leukocytes to produce inflammatory cytokines (mitogen-stimulated) has also been suggested as a possible indicator of inflammation (5). Previous research has shown that physically active individuals (regardless of gender) have a lower plasma inflammatory cytokine concentration, mitogen-stimulated inflammatory cytokines, and hsCRP (5–11). In two recent studies from our laboratory (3,4), we demonstrated that older (65–80 years), physically active women had significantly less lipopolysaccharide (LPS)-stimulated inflammatory cytokine production than did older, sedentary women. Although the relationship between physical activity and inflammatory cytokines has been documented, the mechanism for the response has not been elucidated.

It was hypothesized that the Toll-like receptor 4 (TLR4) pathway may play a role in the anti-inflammatory effects of a physically active lifestyle (4). TLR4 modulates the pathway that allows cells to respond to in vitro LPS stimulation (12,13). In addition to the response to LPS, TLR4 signaling has been shown to be effected by heat shock proteins (HSP 60 and HSP 70) and alterations in mitogen-activated protein kinase (MAPK) activity (14). Cells with a high cell-surface expression of TLR4 produce significantly more inflammatory cytokine when stimulated with LPS (mitogen) than do cells with low TLR4 expression (12,13). In previous studies from our laboratory, we have found that TLR4 expression (messenger RNA and cell-surface) was lower in physically active older women compared to sedentary older women (3,4). These studies support a possible relationship between TLR4 and inflammatory cytokines, which might explain the anti-inflammatory effects attributed to a physically active lifestyle.

Aging has been associated with an accumulation of chronic inflammation (7,8). Our previous investigations have focused on older women (3,4) and have not included men or younger individuals. To our knowledge, no published studies have examined the effect of age and physical activity status in both men and women. We hypothesize that...
older participants will have greater LPS-stimulated inflammatory cytokine production, higher TLR4 expression, and more hsCRP than will younger participants. We further hypothesize that, independent of age; inactive participants will have greater LPS-stimulated inflammatory cytokine production, higher TLR4 expression, and more hsCRP than will active participants. The purpose of this study was to examine the association between physical age, physical activity status, and the degree of chronic inflammation as assessed by inflammatory cytokine production and plasma hsCRP. The association of age and physical activity status on CD14+ cell-surface expression of TLR4 was also examined.

**METHODS**

**Participants**

All testing procedures were approved by the Committee for the Use of Human Subjects at Purdue University. Men and women were recruited to fill one of four groups: old (60–80 years) active lifestyle; old inactive lifestyle; young (18–30 years) active lifestyle; or young inactive lifestyle (Table 1). Participants were screened over the phone prior to the laboratory visit to estimate activity status and rule out any contraindications for the study. After the phone screening, participants reported to the Wastl Human Performance Laboratory and completed a university-approved informed consent, medical history, and a Paffenbarger Physical Activity Questionnaire (15). Participants with a known metabolic disorder (e.g., diabetes mellitus, elevated cholesterol), HIV or AIDS, an autoimmune disease (e.g., arthritis), and/or cardiovascular disease were excluded.

Additional exclusionary criteria included: major surgery in the past year, current or previous (last 12 months) tobacco use, more than a moderate intake of alcohol (>2 drinks per day), current or previous elicit drug use, current use of hormone replacements (e.g., hormone replacement therapy, oral contraceptives, or testosterone), current use of selective estrogen receptor modulators, current use of bis-phosphonate drugs, current use of prescription anti-inflammatory drugs, current use of drugs designed to alter blood cholesterol (e.g., statins), 10% change in body weight over the previous 6 months, and/or a body mass index above 35 kg·m⁻². Participants who consumed over-the-counter anti-inflammatory medications (e.g., ibuprofen, naproxen sodium) and/or aspirin were asked to discontinue its use 7 days prior to blood sampling to allow for washout.

Participants who cleared the initial screening completed a modified Balke submaximal exercise test on the treadmill to predict maximal oxygen consumption (VO2max, an indicator of fitness level; 16). Group assignments were determined by comparing the participant’s VO2max and physical activity questionnaire scores. Inactive participants had a VO2max which was below average for their age group and a sedentary lifestyle. Active participants had a VO2max which was at least above average for their age group and an active lifestyle. If the Paffenbarger scale and estimated VO2max did not indicate the same status for a given participant, that participant was excluded from the study (n = 5). Body composition was determined by bioelectrical impedance analysis (BIA; Omron, Bannockburn, IL). Participants cleared for this analysis were scheduled for a blood sampling trial.

**Presampling Control**

Two days prior to the blood sampling day, participants reported to the laboratory and were given a eucaloric diet to consume the day (24 hours) prior to blood sampling that included three meals plus a snack. Standard mixed diets (50% carbohydrate, 15% protein, and 35% fat) were prepared for each participant by a registered dietician in the Department of Foods and Nutrition at Purdue University. The total energy content of the diet was determined using the Harris–Benedict equations for men and women, based on body weight, height, and age (17). According to the Harris–Benedict equation, different activity factors were incorporated for active (1.7–1.8) and inactive (1.6–1.7) participants. Participants were provided detailed instructions regarding consumption of the control diet and were asked not to exercise 72 hours prior to the blood collection. Blood sampling for all younger women was completed within 7 days after the onset of menses.

**Table 1. Participant Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Old, Active (N = 23)</th>
<th>Old, Inactive (N = 21)</th>
<th>Young, Active (N = 21)</th>
<th>Young, Inactive (N = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>72 ± 5</td>
<td>69 ± 4</td>
<td>24 ± 4.8</td>
<td>24 ± 5.1</td>
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<tr>
<td>Height, cm</td>
<td>167.4 ± 8.1</td>
<td>168.9 ± 7.1</td>
<td>174.0 ± 12.2</td>
<td>171.7 ± 9.1</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>68.9 ± 9.9</td>
<td>79.5 ± 15.7</td>
<td>70.6 ± 11.2</td>
<td>76.5 ± 16.0</td>
</tr>
<tr>
<td>BMI, kg·m⁻²</td>
<td>24.2 ± 2.7*</td>
<td>27.7 ± 4.2</td>
<td>23.3 ± 2.5*</td>
<td>25.9 ± 3.7</td>
</tr>
<tr>
<td>Percent body fat</td>
<td>32.1 ± 7.2*</td>
<td>35.2 ± 7.9</td>
<td>17.3 ± 7.6*</td>
<td>22.9 ± 5.6*</td>
</tr>
<tr>
<td>VO2max, ml·kg⁻¹·min⁻¹</td>
<td>39.1 ± 8.0*</td>
<td>25.0 ± 5.8</td>
<td>47.9 ± 8.7*</td>
<td>31.9 ± 7.3*</td>
</tr>
<tr>
<td>Leukocyte count (×10⁹·L⁻¹)</td>
<td>3.68 ± 1.38</td>
<td>3.95 ± 1.04</td>
<td>4.02 ± 1.48</td>
<td>4.64 ± 1.35</td>
</tr>
<tr>
<td>Monocyte count (×10⁶·L⁻¹)</td>
<td>0.15 ± 0.10</td>
<td>0.21 ± 0.18</td>
<td>0.17 ± 0.14</td>
<td>0.11 ± 0.05</td>
</tr>
<tr>
<td>Estradiol, pg·mL⁻¹</td>
<td>24.3 ± 10.9</td>
<td>22.1 ± 16.0</td>
<td>39.8 ± 21.1*</td>
<td>66.2 ± 22.0*</td>
</tr>
<tr>
<td>Testosterone, pg·mL⁻¹</td>
<td>4.30 ± 1.05</td>
<td>3.86 ± 2.10</td>
<td>4.95 ± 0.96</td>
<td>4.17 ± 0.91</td>
</tr>
<tr>
<td>Daily physical activity, h-day⁻¹</td>
<td>2.7 ± 0.8*</td>
<td>0.5 ± 0.6</td>
<td>2.7 ± 0.7*</td>
<td>0.4 ± 0.5</td>
</tr>
</tbody>
</table>

Notes: Values represent mean ± standard deviation.
*Different than inactive groups (p < .05).
**Different than old groups (p < .05).
† Estradiol analysis was only completed on female participants, testosterone analysis was only completed on male participants.
Blood Sampling Day
Participants arrived at the laboratory between 6:00 AM and 8:00 AM following an overnight fast (>8 hours) and rested quietly in a seated position for 30 minutes prior to blood sampling. Venous blood samples were collected into evacuated tubes (Vacutainer; Becton-Dickenson, Franklin Lakes, NJ) by a trained technician using a butterfly needle. Vacutainer tubes were pretreated with EDTA (plasma and flow cytometry), sodium heparin (LPS stimulation), or a silica and gel (SST) clot activator (serum). Plasma and serum were separated from blood cells within 30 minutes of collection and stored at −80°C prior to analysis for testosterone, estrogen, and hsCRP.

Leukocyte Counts
An average of triplicate leukocyte counts was determined as described previously (3,4) using an automated method (Z2 Coulter Counter; Beckman-Coulter, Miami, FL). The intra- and inter-assay coefficients of variation were less than 5% for all analyses. Monocyte count was determined by multiplying percent monocyte (determined by primary flow cytometry gating) by total leukocyte count.

Flow Cytometry
Immunocytometry analysis was performed as described previously (4,18). Briefly, aliquots (100 µl) of EDTA-treated whole blood were combined with two different monoclonal-fluorescent labeled antibody cocktails in the bottom of separate polystyrene 12 × 75 mm tubes (Sarstedt, Newton, NC). In Tube 1, the blood was combined with: CD14-FITC (Beckman-Coulter) and TLR4-PE (eBioscience, San Diego, CA). Tube 2 was labeled with immunoglobulin G (IgG)-FITC (e-bioscience) and IgG-PE (eBioscience) to serve as negative controls. Following incubation in the dark (30 minutes), tubes were processed using an automated system (TQ-prep; Beckman-Coulter).

Primary gates were established for monocytes based on forward and 90° side scatter. Secondary gates were established for CD14 within the primary monocyte gate. TLR4 mean fluorescent intensity (MFI) was determined on CD14+ cells. Negative regions were set daily using isotype control tubes. All sample analyses were completed on an FC500 flow cytometer equipped with a 488 nm air-cooled argon laser (Beckman-Coulter).

In Vitro Cytokine Stimulation and Production
LPS-stimulated production of IL-6, IL-1β, and TNF-α was determined using a whole-blood technique described previously (3,4). Briefly, sodium heparin–treated whole blood was diluted (1:20 in RPMI 1640, supplemented with L-glutamine [2 mM] and penicillin [100 U·mL⁻¹]/streptomycin [100 µg·mL⁻¹]), transferred to separate wells of a 24-well microplate, combined with LPS from Salmonella enteriditis (final concentration: 25 µg·mL⁻¹), Sigma-Aldrich, St. Louis, MO), and incubated in a 37°C, 5% CO₂ environment for 24 hours. Final concentration of LPS was determined by a lot-specific titration curve which was completed prior to the study (data not shown). The dose of LPS selected was chosen to elicit maximal cytokine production. Following incubation, cell-free supernatants were collected and frozen at −80°C until analysis.

Culture supernatant was diluted 1:80 (IL-6), 1:40 (IL-1β), and 1:10 (TNF-α) with assay diluent (10% fetal bovine serum in phosphate-buffered saline) to determine LPS-stimulated cytokine concentrations using separate enzyme-linked immunosorbent assay (ELISA) sets (BD Biosciences, Franklin Lakes, NJ). Optimal supernatant dilution factor was determined previously in our laboratory (3,4). Coefficients of variation (intra- and inter-assay), established using an internal laboratory control, were less than 5% for all analyses. To minimize variation, all samples for a given analyte were analyzed on the same day.

Plasma and Serum Analyses
EDTA-treated plasma was analyzed for total testosterone (male participants only) and estrogen (female participants only), and serum was analyzed for hsCRP using separate commercially available ELISA kits (Alpco Diagnostics, Windham, NH).

Statistical Analyses
Prior to statistical testing, data were assessed to determine that assumptions for normality and constant variance were met using quantile–quantile and residual plots, respectively. All dependent variables were analyzed using a 2 (age: old and young) × 2 (physical activity status: active and inactive) analysis of variance (ANOVA) using PC-SPSS (version 12.0; Chicago, IL). Significance for all tests was set at p < .05. When significant interactions were detected, location of significance was determined using separate Student t tests with a Bonferroni correction for multiple comparisons. All dependent variables are presented as mean ± standard error of the mean (SEM). Correlation analysis was completed on the key dependent variables in the present study to further explore the relationship between age, activity status, biomarkers of inflammation, and TLR4.

RESULTS

Leukocyte Counts
No significant differences were found for leukocyte or monocyte counts (Table 1).

Flow Cytometry
No significant differences were found for CD14+ count (Figure 1A). A significant main effect for physical activity status was found for TLR4 (F = 10.722, p = .002; Figure 1B), where inactive was 32% greater than active.

In Vitro Cytokine Stimulation and Production
A significant main effect for physical activity status was also found for LPS-stimulated IL-6 production (F = 10.139, p = .002; Figure 2A), where inactive was 24% greater than active. A significant Age × Physical Activity Status interaction was found for LPS-stimulated IL-1β (F = 5.380, p = .023, Figure 2B). Young-active was 44% higher than old-active, and old-inactive was 14% higher than young-active. Finally, a significant main effect for physical activity
status was found for LPS-stimulated TNF-α production ($F = 6.039, p = .016$; Figure 2C), where inactive was 21% higher than active.

**Plasma and Serum Analyses**

No significant effects were found for plasma testosterone (male participants only, Table 1). A significant main effect for age was found for plasma estradiol (female participants only, $F = 5.437, p = .029$; Table 1), where young participants had 44% more estradiol than old participants had. Significant main effects for age ($F = 8.809, p = .004$) and physical activity status ($F = 5.942, p = .017$) were found for serum hsCRP (Figure 3). Specifically, old participants had 53% higher hsCRP than did young participants, and active participants had 60% lower hsCRP than did inactive participants.

**Correlation Analysis**

Correlations were completed to compare the nature of the association between physical age, inflammatory biomarkers, and TLR4 by dividing the participants based on activity status (i.e., active vs inactive). For active participants, we found that age was significantly correlated to hsCRP (0.361, $p = .022$), LPS-stimulated IL-6 production (−0.327, $p = .032$), and LPS-stimulated IL-1β production (−0.374, $p = .013$). Similar correlation trends existed for inactive participants; however, they were not significant. Correlations were also completed with all participants in a single analysis. These analyses revealed that daily physical activity was significantly correlated to LPS-stimulated IL-6 production (−0.279, $p = .011$), LPS-stimulated IL-1β production (−0.232, $p = .036$), and TLR4 cell-surface expression (−0.234, $p = .035$). The nature of these negative correlations suggests that as daily physical activity increases, LPS-stimulated IL-6, LPS-stimulated IL-1β, and TLR4 decrease.

**DISCUSSION**

The key findings of this investigation were that physically inactive participants had higher CD14 cell-surface expression of TLR4 and higher LPS-stimulated inflammatory cyto-
We demonstrated that physically active participants (regardless of age) had less cell-surface TLR4 expression than did physically inactive participants; however, we did not find a significant association with age. Also, we found that TLR4 expression was positively correlated to LPS-stimulated inflammatory cytokine production. These findings are consistent with previous reports from our laboratory and others (3,4,12,13). Also, TLR4 expression mirrored the inflammatory cytokine production response, where physical activity status, but not age, was associated with the response. The lack of age association may suggest that TLR4 expression is mediated by a mechanism similar to that of inflammatory cytokine production and is not directly related to hsCRP. More research is needed to fully evaluate the potential association between hsCRP and TLR4.

The key purpose of this study was to examine the association between various inflammatory biomarkers and TLR4 in individuals of different age and activity status. We selected an array of inflammatory markers which were assessed from various tissue sources (i.e., plasma, serum, and whole blood) which may complicate the comparison of the responses. We acknowledge that it may be difficult to fully compare all of the markers measured due to differences in measurement technique and the physiological source of the biomarkers.

**Conclusion**

The key findings of the present study were that physically active participants had significantly lower hsCRP levels, inflammatory cytokine production, and cell-surface TLR4 expression than did physically active participants. Quantity of daily physical activity was also negatively correlated to LPS-stimulated cytokine production (IL-6 and IL-1β) and TLR4. The findings of the present study support the hypothesis that regular physical activity elicits anti-inflammatory effects, which may lower chronic inflammation. In addition, the fact that the response of hsCRP, inflammatory cytokine production, and TLR4 expression differed suggests that they may be regulated by different mechanisms. Additional research is needed to identify and evaluate which exercise-mediated mechanisms regulate biomarkers associated with chronic inflammation.

**REFERENCES**


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