Voluntary wheel running attenuates lipopolysaccharide-induced liver inflammation in mice

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1Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada; 2Departments of Nutrition and Exercise Physiology, University of Missouri, Columbia, Missouri; and 3Department of Medicine, University of Missouri, Columbia, Missouri; and 4Research Service-Harry S. Truman Memorial VA Hospital, Columbia, Missouri

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Peppler WT, Anderson ZG, Sutton CD, Rector RS, Wright DC. Voluntary wheel running attenuates lipopolysaccharide-induced liver inflammation in mice. Am J Physiol Regul Integr Comp Physiol 310: R934–R942, 2016. First published February 17, 2016; doi:10.1152/ajpregu.00497.2015.—Sepsis induces an acute inflammatory response in the liver, which can lead to organ failure and death. Given the anti-inflammatory effects of exercise, we hypothesized that habitual physical activity could protect against acute sepsis-induced liver inflammation via mechanisms, including heat shock protein (HSP) 70/72. Male C57BL/6J mice (n = 80, ~8 wk of age) engaged in physical activity via voluntary wheel running (VWR) or cage control (SED) for 10 wk. To induce sepsis, we injected (2 mg/kg ip) LPS or sterile saline (SAL), and liver was harvested 6 or 12 h later. VWR attenuated increases in body and epididymal adipose tissue mass, improved glucose tolerance, and increased liver protein content of PEPCK (P < 0.05). VWR attenuated increases in LPS-induced IL-6 signaling and mRNA expression of other inflammatory markers (TNF-α, chemokine C-C motif ligand 2, inducible nitric oxide synthase, IL-10, IL-1β) in the liver; however, this was not reflected at the whole body level, as systemic markers of inflammation were similar between SED and VWR. Insulin tolerance was greater in VWR compared with SED at 6 but not 12 h after LPS. The protective effect of VWR occurred in parallel with increases in the liver protein content of HSP70/72, a molecular chaperone that can protect against inflammatory challenges. This study provides novel evidence that physical activity protects against the inflammatory cascade induced by LPS in the liver and that these effects may be mediated via HSP70/72.

SEPSIS IS A CONTINUUM OF CLINICAL events defined by the presence of infection and systemic inflammation (18). The worldwide incidence of sepsis was recently estimated at 437 cases per 100,000 person years between 2003 and 2015, with an associated fatality rate of 17% (11). This puts a significant burden on the health care system, and in the United States alone, the direct hospital costs associated with sepsis are over $24 billion (17).

The infiltration of a host with both gram-negative and gram-positive bacterial species is often considered to be the cause of acute sepsis (34). LPS from Escherichia coli, a component of gram-negative bacteria, is often used to produce these effects in rodent models. Upon exposure to LPS, an inflammatory cascade is activated both systemically and at a tissue-specific level (1). Initial signaling involves a Toll-like receptor 4 (TLR4)-myeloid differentiation primary response gene 88 (MyD88) interaction that then propagates the inflammatory response (24). Increases in circulating inflammatory factors, such as TNF-α, IL-6, and IL-1β, occur rapidly and are regulated by the dose and duration of exposure (21, 29, 40). Clearance of inflammatory markers begins as early as 8 h postexposure, and most markers return to baseline levels by 16 h (10). In parallel with the systemic inflammatory response, acute exposure to LPS induces profound inflammation within the liver. LPS activates JAK, a STAT signaling complex, which includes phosphorylation of STAT3, and is a well-known marker of IL-6 signaling (31, 36, 38). Further, SOCS3 is induced by activation of the JAK/STAT signaling cascade and acts as an inhibitor of this pathway (6). This inflammatory cascade leads to increases in circulating markers of liver damage, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (12), and morphological alterations to the liver (39).

A number of different mechanisms have been shown to modulate the inflammatory response to LPS-induced inflammation. Of interest, the transforming growth factor β (TGF-β) superfamily protein follistatin (FST) offers protection via binding to activin A (16, 37), proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibits LPS uptake and clearance (35), and heat shock protein (HSP) 70/72 acts a molecular chaperone (2, 8, 23). Since physical activity, in the form of voluntary wheel running (VWR), leads to increases in the protein content of HSP70/72 in skeletal muscle (14), VWR may be able to protect against LPS-induced inflammation. Somewhat surprisingly, Martin et al. (22) demonstrated that 10 wk of VWR did not protect against increases in the hepatic expression of TNF-α, IL-6, and IL-1β when assessed 24 h after a 0.33 mg/kg injection of LPS. However, at 24 h post-LPS, others have shown circulating inflammatory markers are at near-baseline levels (10), and this dose of LPS leads to less than peak inflammatory responses (21). With these points in mind, a protective effect of physical activity may have been missed.

In this report, we assessed whether VWR could protect against LPS-induced liver inflammation in C57BL/6J mice at 6 and 12 h after a 2 mg/kg injection, which we consider to correspond to late peak and early resolution of the inflammatory response (10, 21). We hypothesized that VWR would protect against the deleterious effects of LPS on the liver and that this would be associated with increases in HSP70/72 and follistatin and reductions in PCSK9. Indeed, here, we show that VWR provides modest protection against the inflammatory cascade induced by LPS within the liver at both 6 and 12 h postexposure and that this occurs, in parallel with increases in HSP70/72.
METHODS

Ethics. All procedures adhered to the guidelines of the Canadian Council on Animal Care and were approved by the University of Guelph Animal Care Committee.

Animals. Eight-week-old male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These experiments were completed in two rounds of n = 40 each. In round 1, mice were exposed to saline (SAL) or LPS for 6 h, and in round 2, mice were exposed to SAL or LPS for 12 h. After acclimation, mice were housed in shoebox cages as sedentary controls (SED group; n = 20 per experiment) or in cages outfitted with a 14-cm diameter running wheel (VWR group, n = 20 per experiment) and rotation counter (VDO M3 wired bike computer; Mountain Equipment Co-Op, Vancouver, Canada). All mice were housed individually on a 12:12-h light-dark cycle (~9 AM to 9 PM), with access to food and water ad libitum. Body mass and food intake (amount of food in hopper of cage) were measured weekly.

Glucose tolerance test. During the final week of the intervention, and at least 48 h prior to LPS injections, an intraperitoneal glucose tolerance test (GTT) was performed. Following a 6-h fast, and with wheels removed from cages, mice were injected intraperitoneally with a weight-adjusted bolus (2 g/kg) of d-glucose. Tail blood glucose was measured immediately prior to the glucose injection and at 15, 30, 45, 60, 90, and 120 min afterward using a hand-held Freestyle Lite glucometer (Abbott Laboratories; Abbott Park, IL). The glucose area under the curve (AUC) was then calculated.

LPS. LPS was purchased from Sigma-Aldrich (St. Louis, MO) (0111-B4, L2630). After 10 wk of VWR or SED control, a dose of 2 mg/kg of LPS was injected intraperitoneally into awake, nonanesthe-
tized mice starting at 9 or 6 AM (6- and 12-h experiments, respec-
tively), and control mice were injected with SAL. A 2 mg/kg injection of LPS has been shown to induce peak cytokine concentrations in mice (30). To assess LPS-mediated impairment of glucose homeostasis, and levels of gluconeogenic enzymes previously described (19, 28). Measurement of TNF-α, IL-6, IL-1β, Ccl2, Nos2, IL-10, SOCS3 (Mcytomag-70K-06), and FST (MAGPMAG-24K-01) were completed using the Milliplex MAP system (Billerica, MA). Samples were diluted twofold, and for IL-6 and Ccl2, data points that fell above the standard curve were extrapolated using the five-parameter logistic curve-fitting method.

Statistical analysis. Data were analyzed using repeated-measures two-way ANOVA (GTT), three-way ANOVA (ITT), unpaired t-test (body mass, AUC, and fold changes), and two-way ANOVA (all other measures). Data were first assessed for normality and homogeneity of variance, and when not met for two-way ANOVA tests, the data were transformed (log10). For t-tests a Mann-Whitney U-test or Welch’s correction was used. The uncorrected Fisher’s least significant difference post hoc test was used to compare two-way ANOVA interactions. All data are presented as means ± SE, and a significance level of P < 0.05 was used. Statistical tests were completed using SigmaPlot version 11.0 (San Jose, CA) and Graph Pad version 6.2 (La Jolla, CA).

RESULTS

VWR attenuates body mass and adipose tissue gain, improves glucose tolerance, and increases markers of hepatic gluconeogenesis. We first wanted to ensure the effectiveness of VWR; thus, we assessed differences in body mass, glucose homeostasis, and levels of gluconeogenic enzymes previously shown to be increased with exercise (13). Mice in the VWR group averaged ~3–5 km/night over the 10-wk period (Fig. 1A), and despite greater food intake than SED mice (Fig. 1B), they had attenuated gains in body mass (Fig. 1C). VWR also improved glucose tolerance (Fig. 1D) and reduced epididymal white adipose tissue mass (Fig. 1E). Further, the protein

Table 1. TaqMan PCR primers

<table>
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<tr>
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All expression assays were purchased from Thermo Fisher Scientific (cat. no. 4331182).
we compared the level of induction only between LPS-injected mice after LPS; however, as this was undetectable in SAL controls, A and post-LPS (two-way ANOVA main effect, \( P < 0.05 \)). In mice injected with LPS, the phosphorylation of STAT3 was increased induction of IL-6 signaling after LPS exposure. In mice injected with LPS and found that VWR mice had an attenuated increase in IL-1\( \alpha \), H9251, IL-6, Ccl2, Nos2, and IL-10 \( ( P < 0.001 \) ) (Fig. 2A–F, insets). At 12 h post-LPS, VWR mice injected with LPS had an attenuated increase in IL-1\( \beta \) compared with SED mice \( ( P = 0.002 \) ) (Fig. 3C). In addition, there was a main effect of VWR for TNF-\( \alpha \), IL-1\( \beta \), and Ccl2 (Fig. 3A, C, D). When comparing the level of induction above SAL control within SED and VWR mice injected with LPS and found that VWR mice had attenuated increases in TNF-\( \alpha \), IL-6, Ccl2, Nos2, and IL-10 \( ( P < 0.05 \) ) (Fig. 2A, C, D, and E). Together, these data suggest that VWR attenuates inflammatory response to LPS within the liver when assessed at both 6 and 12 h post-LPS.

Next, we wanted to determine whether VWR attenuated the induction of IL-6 signaling after LPS exposure. In mice injected with LPS, the phosphorylation of STAT3 was increased \( ( P < 0.001 \) ), which was modestly attenuated by VWR at 12 h post-LPS (two-way ANOVA main effect, \( P < 0.001 \) ) (Fig. 4A, A and B). The phosphorylation of STAT1 was also increased after LPS; however, as this was undetectable in SAL controls, we compared the level of induction only between LPS-injected mice. At 6 h post-LPS, there was no protective effect of VWR against increases in the phosphorylation of STAT1 \( ( P = 0.95 \) ); however, at 12 h post-LPS, VWR offered mild protection \( ( P = 0.10 \) ) (Fig. 4C). In contrast, the fold change compared with SED control was attenuated at both 6 and 12 h post-LPS by VWR \( ( P < 0.05 \) ) (Fig. 4E). These results did not translate to the protein level, as the content of hepatic SOCS3 was not different with VWR or LPS (data not shown).

To determine whether the beneficial effect at the mRNA and protein level in the liver corresponded to clinically relevant markers of liver damage, we measured serum AST and ALT, and both increased in response to LPS (two-way ANOVA main effect of LPS, \( P < 0.001 \) ). However, this increase was similar in SED and VWR mice for AST (SED SAL: 94.4 ± 11.3 U/l; VWR SAL: 97.7 ± 9.4 U/l; VWR LPS: 185.8 ± 18.7 U/l) and ALT (SED SAL: 25.9 ± 2.8 U/l; VWR SAL: 21.8 ± 1.2 U/l; SED LPS, 41.8 ± 1.0 U/l; VWR LPS: 50.0 ± 8.7 U/l). As further confirmation of this finding, we wanted to determine whether LPS exposure led to morphological alterations in the liver and whether VWR could protect against this. There were no gross histological alterations observed (data not shown).

We next wanted to determine whether the attenuation of LPS-induced liver inflammation occurred in parallel with attenuated levels of the circulating inflammatory markers IL-6, Ccl2, IFN-\( \gamma \), TNF-\( \alpha \), IL-1\( \beta \), and IL-10. There was a significant induction of all inflammatory markers in mice injected with LPS; however, as the levels in mice injected with SAL were below the level of detection, we compared SED and VWR

Fig. 1. Effects of voluntary wheel running on distance run (A), food intake (B), body mass (C), blood glucose during a GTT (D), epididymal adipose tissue mass (E), and protein content of PEPCK and G6Pase (F). Values are pooled from 6- and 12-h experiments, unless indicated. Two-way ANOVA main effect for VWR, \( \$ P < 0.05 \), main effect of time. Unpaired t-test between sedentary (SED) and voluntary wheel running (VWR) rats, \( * P < 0.05 \).
The protective effects of voluntary wheel running on LPS-induced inflammation are associated with increases in HSP70/72. As VWR conferred a protective effect against LPS induced inflammation in the liver, we next sought to elucidate potential mechanisms that could be mediating this effect. First, we assessed members of the LPS signaling complex and found no differences in TLR4 protein content with VWR or LPS (Fig. 6A); however, MyD88 protein content was increased at both 6 and 12 h post-LPS (−1.7 and 1.3 fold, respectively), which was similar between SED and VWR groups (Fig. 6B). Next, we assessed hepatic FST and found that the mRNA expression was robustly elevated in mice injected with LPS. At 6 h post-LPS, VWR attenuated the LPS response compared with
LPS-mediated changes in glucose homeostasis are modestly altered by voluntary wheel running. As a final outcome measure, we wanted to determine whether VWR could protect against LPS-induced hypoglycemia and insulin resistance. At both 6 and 12 h post-LPS, blood glucose values were reduced to ~40% of SAL control, with no protective effect of VWR (data not shown). Upon insulin injection, the blood glucose response (i.e., reduction) was impaired in mice injected with LPS, and when comparing the AUC of the blood glucose response VWR moderately attenuated the impairment at 6 but not 12 h post-LPS (main effect of VWR, \( P = 0.004 \)) (Fig. 7, A and B). Together, this suggests that VWR is not able to offer significant protection against LPS-mediated reductions in blood glucose and insulin resistance, especially with a prolonged exposure to LPS.

**DISCUSSION**

In this study, we demonstrate that 10 wk of VWR attenuates the inflammatory response to LPS within the liver of male C57BL/6J mice. In a time-dependent manner, VWR led to attenuations in the liver mRNA expression of known inflammatory markers (24). At 6 h post-LPS, there were attenuations in IL-6 and FST, while at 12 h post-LPS, IL-1β was attenuated, which together is indicative of a reduced inflammatory load. These effects occurred despite only minor alterations to circulating levels of inflammatory markers, which supports the idea that the protective effect of VWR was directly in the liver. In addition, there was modest prevention of insulin resistance in response to LPS in VWR mice at 6 h post-exposure, but not at 12 h post-exposure. Despite these protective effects, VWR had

**Fig. 4.** Effect of voluntary wheel running and LPS on the phosphorylation of STAT3 (A) and STAT1 (B) and the mRNA expression of SOCS3 (C). Two-way ANOVA \( P \) values are indicated below figures, with post hoc testing of interaction. \( \dagger \) \( P < 0.05 \) indicates LPS different within SED or VWR. \( \ddagger \) \( P < 0.05 \) indicates VWR different within SAL or LPS group. *Inset: fold change compared with SAL control. \( ^* P < 0.05 \) for \( t \)-test between SED and VWR groups.

SED mice, and at 12 h post-LPS, there was a two-way ANOVA interaction that revealed the induction of FST post-LPS was lower in VWR mice (\( P = 0.029 \)) (Fig. 6C). Circulating levels of FST, although induced by LPS, did not differ between SED and VWR mice (Fig. 6D). Further, there were no differences in the hepatic protein content of FST with VWR or LPS (data not shown). Third, we measured PCSK9, and neither VWR nor LPS had an effect (Fig. 6E). Lastly, we measured the protein content of HSP70/72 and in both the 6- and 12-h experiments, higher levels of HSP70/72 were observed in VWR mice regardless of SAL or LPS treatment (6-h main effect of VWR, \( P < 0.01 \); 12-h main effect of VWR, \( P < 0.001 \)) (Fig. 6F).
little influence on AST and ALT, which likely indicates that VWR is not able to overcome the robust effect of a supraphysiological LPS challenge on these measures.

The effects of VWR on modulating LPS-induced liver inflammation have produced mixed results. For example, Martin et al. (22) used a 0.33 mg/kg injection of LPS and did not show a protective effect of VWR on increases in the liver mRNA expression of inflammatory markers TNF-α, IL-6, and IL-1β when assessed at 24 h post-LPS. In contrast, here, we show a protective effect of VWR on the mRNA expression of IL-6 at 6 h post-LPS, and for IL-1β at 12 h post-LPS. In support of our data, others have demonstrated that 4 wk of treadmill exercise attenuates the deleterious effects of a 10 mg/kg injection of LPS on mean arterial pressure, heart rate, and leukocytopenia in non-anesthetized rats over 72 h (4). As we used a 2 mg/kg injection of LPS, which has been shown to induce a peak inflammatory response (21), but does not lead to significant mortality, as would higher doses (32), and measured mRNA expression in the liver at time points prior to significant resolution of the systemic inflammatory response (10), the discrepancies between our study and that by Martin et al. (22) are likely attributed to dose and time. However, the protective effect that we observed for the mRNA expression of inflammatory markers was not found for protein content (e.g., pSTAT3). This may be due to a different time or dose response in the liver at the protein level vs. that systemically (10).

In addition to liver, it is possible, that VWR may also confer a protective effect against LPS-induced markers of inflamma-
time points at ANOVA interaction post hoc indicates LPS different from SED for indicated baseline levels. Inset indicates area under curve (AUC). †A three-way SAL-injected (circles) and LPS injected (squares), and data were normalized to after LPS injections. Insulin tolerance test (ITT) curve shows B Fig. 7. Time course of blood glucose response to an insulin injection at 6 h (A) and 12 h (B) after LPS injections. Insulin tolerance test (ITT) curve shows SAL-injected (circles) and LPS injected (squares), and data were normalized to baseline levels. Inset indicates area under curve (AUC). †A three-way ANOVA interaction post hoc indicates LPS different from SED for indicated time points at P < 0.001.

In this study, we were interested in determining potential mechanisms through which VWR may protect against LPS induced inflammation. In VWR mice, we detected increases in the liver protein content of HSP70/72, a protein considered to have dual roles as a molecular chaperone and in buffering against metabolic stressors (2). Unlike other studies that observed increases in HSP70/72 protein content in the skeletal muscle after LPS exposure (7), we did not observe an effect of LPS in the liver but only of VWR. Increases in the liver protein content of HSP70/72 have recently been shown to protect against LPS induced inflammation. For example, adenoviral overexpression of HSP70/72 in the liver attenuated LPS-induced increases in serum IL-6, serum TNF-α, and hepatic NF-κB p65 expression in the nucleus (8). In addition, others (23) have used heat stress to induce HSP72 prior to an acute injection (10 mg/kg) of LPS in normal or cirrhotic rats, and this protected against increases in AST, ALT, and TNF-α in serum, while also preventing neutrophil infiltration in the liver. Together, our data, and that from previous studies (8, 23), suggest that HSP70/72 likely contributes to the beneficial effect of VWR on LPS-induced inflammation.

In addition to HSP70/72, we were also interested in exploring the potential role of the TLR4-MyD88 signaling complex and FST in mediating the protective effects of VWR. While others have shown that swim training reduces the protein content of TLR4 and its association with MyD88 in livers from rats fed a high-fat diet (26), we did not detect reductions in the content of these proteins in response to LPS injection in mice given access to a running wheel. Next, we wanted to determine the role of FST, a member of the TGFβ superfamily that has been shown to confer protection against LPS-induced inflammation. FST mRNA expression in the liver and circulating levels peak ~5 h post-exposure to LPS (37), and the direct treatment of mice with FST prior to LPS exposure attenuates the inflammatory response (16). We show that FST mRNA in the liver is increased at 6 and 12 h post-LPS and that this is moderately attenuated with VWR. These findings suggest that despite a response of FST to LPS, it likely does not play a role in the beneficial effects of VWR. As FST would appear to be increased in response to inflammatory stresses, the blunted induction of FST in liver from VWR mice in our study may be the result of the attenuated induction of liver inflammation in these animals.

Perspectives and Significance

In this study, we demonstrate that habitual physical activity, via 10 wk of VWR, is able to protect against the inflammatory cascade induced by LPS within the liver of mice. The protective effect is primarily evident for mRNA expression of inflammatory cytokines and occurred in parallel with increases in the protein content of HSP70/72. Future work should explore whether the protective effect of habitual physical activity occurs across different doses and durations of LPS exposure and if a similar relationship exists with HSP 70/72 in different tissues.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


