Salmonella administration induces a reduction of wheel-running activity via a TLR5-, but not a TLR4, dependent pathway in mice

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ABSTRACT

In general, systemic bacterial infections induce sickness behavior. In mice, lipopolysaccharide (LPS), a component of gram-negative bacteria, strongly reduces physical activity via toll-like receptor (TLR) 4. However, gram-negative bacteria, such as Salmonella, also express flagella containing flagellin (FG) which binds to TLR5 and induces pro-inflammatory cytokine production. It is unclear whether FG induces sickness behavior. To determine whether Salmonella administration regulates the reduction of voluntary physical activity in mice, male C3H/HeN (wild type) and C3H/HeJ (tlr4 gene mutated) mice were administered living Salmonella (live) and examined for wheel-running activity. The production of TNF-α in RAW 264 cells was measured by the ELISA assay under both live and heat-killed (HK) Salmonella conditions in vitro. Wheel-running activity in both C3H/HeJ and C3H/HeN mice after i.p. injection of live Salmonella ($1x10^6$ CFU/kg) was significantly lower than that in vehicle groups ($p<0.01$, respectively), although wheel-running activity in C3H/HeJ mice was not reduced after i.p. injection of HK Salmonella ($1x10^6$ CFU/kg). Furthermore, TNF-α production from RAW 264 cells with HK Salmonella treatment at the early phase was higher than that with live Salmonella treatment. Interestingly, gentamicin-treated (GMT) Salmonella, (which have bacterial flagella removed), did not induce reduction of wheel-running activity, although injection of the flagella-rich supernatant of GMT Salmo-

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nella significantly reduced it ($p<0.01$). Indeed, FG treatment also induced reduction of wheel-running activity in mice ($p<0.01$). Our findings suggest that the Salmonella-induced reduction of voluntary physical activity might be regulated by FG via TLR5, but not LPS via TLR4 in mice.

Keywords: Toll-like receptors TLR4, TLR5, voluntary physical activity, flagellin, lipopolysaccharide, C3H/HeJ mouse

INTRODUCTION

Salmonella, which is a gram-negative bacterium, can invade and cause enteritis, systemic infection and fever (3), and it possesses a range of protein [e.g. lipoprotein and flagellin (FG)] and nonprotein [e.g. lipopolysaccharide (LPS), peptidoglycan (PGN) and CpG DNA] structures that function as pathogen-associated molecular patterns (PAMPs) (1). These PAMPs are recognized by the family of toll-like receptor (TLRs) on host mammalian cells which signal host cells to induce a response (1). It is well known that LPS, a component of the cell wall of gram-negative bacteria (34), is a main mediator of pro-inflammatory cytokine production (1) and quickly induces a range of sickness behaviors in animals (12,17,19,45). LPS typically consist of lipid A, inner and outer cores, and O-antigen, which is the main component of the outer leaflet of the outer membrane of *Salmonella* (32,35). However, the role of LPS versus other PAMPs in the induction of sickness behavior and pro-inflammatory cytokines induced by living bacterium such as *Salmonella* has not been systematically investigated. In fact, Royle et al. (37) reported that administration of a lipid A antagonist prior to live *Salmonella* exposure had no effect on tumor necrosis factor (TNF)-α release from macrophages. In addition, it is also known that changes in gene expression are generally greater in cells treated with LPS than in those infected by living bacteria (36,37). Bacteria of the *Salmonella* family produce a number of specialized effector proteins that can modify host cell signaling (14). Furthermore, the lipid A portion of LPS, which is responsible for the majority of immunomodulating activity of LPS (27,30), is not expressed on outer bacterial membranes, because lipid A locates between PGN and the outer cores/O-antigen in the *Salmonella* cell wall (32,34,35). Therefore, it might be hard for host cells to recognize lipid A in the LPS of living bacteria via TLR4. Accordingly, we hypothesized that PAMPs other than LPS might contribute to behavioral changes following bacterial infection.

FG (the major structural protein of flagella of gram-negative bacteria) has recently been appreciated as a major factor contributing to the host inflammatory response to bacteria (9,13,23). FG induces an inflammatory and innate immune response through activation of TLR5 and is known to be essential for the pathogenesis of many gastrointestinal, respiratory and renal tract bacteria (16). Signaling from FG/TLR5 as well as LPS/TLR4 activates the MyD88 dependent pathway, which sequentially activates IL-1R-associated kinase (IRAK), TNFR-associated factor 6 (TRAF6), nuclear factor kappa B (NF-κB), resulting in the induction of genes involved in inflammatory responses (1). Although factors that regulate sickness behavior via TLRs are poorly understood, pro-inflammatory cytokines [such as interleukin (IL)-1β, IL-6 and TNF-α], prostaglandin and other mole-

cules, which are triggered by NF-κB activation are known to play a role in the behavioral partiality effect of infection (6,7,15,45). In fact, it was reported that intravenous FG caused a systemic inflammatory response (10,46). Therefore, in addition to LPS, it is possible that FG may contribute to the reduction of physical activity following gram-negative bacterial infection. Our hypothesis was that FG, interacting through TLR 5, contributes to the reduction in voluntary wheel-running behavior following *Salmonella* infection. The purpose of the present study was to determine the components of *Salmonella* that regulate the reduction of voluntary physical activity in mice.

MATERIALS AND METHODS

Animals

Male 8-9-week-old C3H/HeN (wild type, n = 55) and C3H/HeJ (*tlr4*-gene mutation, n= 72) mice (Clea Japan, Tokyo, Japan) were used in these experiments. The mice were housed individually in cages with a running wheel (10 x 23 x 10 cm cage with 5.5 wide x 22 cm ø wheel, Natsume, Nagano, Japan) that was accessible 24 hours per day. The animals were under a controlled environment (20 ± 1˚C, 12:12-h light-dark cycle) and allowed unrestricted access to standard chow and tap water. The experimental procedures followed the guiding principles for the care and use of animals in the field of physiological sciences approved by the Council of the Physiological Society of Japan.

Cell culture

RAW 264 cells, a mouse-derived macrophage cell line, were obtained from the Cell Bank RIKEN Bioresource Center (Ibaraki, Japan). These cells were cultured in DMEM containing 10% FCS supplemented 200 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2.

Bacteria and their treatment

Salmonella enterica (serovar Dublin) was provided by Professor Hiroko Mine of the Department of Clinical Nutrition, Kawasaki University of Medical Welfare. The bacteria were grown for 48 h at 35˚C in a brain heart infusion agar (Nissui, Tokyo, Japan) and diluted in sterile physiological saline as live bacteria (Live, $1\times10^{3\text{-}5}$ CFU/ml) or as heat-killed (HK, $1\times10^{3\text{-}5}$ CFU/ml) *Salmonella*, which were treated for 2 h at 62˚C. In some experiments, live *Salmonella* were treated for 1 hr with gentamicin (GM, 100 μg/ml) in RPMI 1640 medium at 35°C. After GM treatment, the medium containing *Salmonella* was centrifuged at 13,000 g for 5 min and then the supernatants were filtered through a 0.22 μm membrane to remove bacteria and or any cellular debris. The purity was confirmed by SDS-PAGE and Coomassie blue staining. The flagella-based motility of *Salmonella* was examined by a motility test. Briefly, *Salmonella* were oscillating-cultured for 5 hr with brain heart infusion medium containing 0.3% agar at 35ºC (39). Western blot analysis was performed to examine flagellin (FG) content of GM treated *Salmonella* and supernatant of GM treated *Salmonella*. Briefly, following electrophoresis, the gel was transferred to a cellulose nitrate membrane filter and excessive proteins in the uncombined part of the membranes were saturated with

3% BSA/TBS (bovine serum albumine/tris–buffered saline, pH 7.5) overnight at 4˚C. Then the membrane was soaked in purified anti-Flagellin mouse IgG1 antibody (Biolegend, San Diego, CA) with 1%BSA/TBS solution overnight at 4˚C (1:500). After the membranes were washed, they were soaked in POD-linked goat IgG to mouse IgG (Nordic Immunological, Tilburg, Netherlands) with 1%BSA/TBS solution for 1 hr at room temperature (1:1,000). After the membrane were washed again, they were stained with 6 μl 30% H_2O_2/10 ml TBS including 6 mg 4-chloro-1-naspthol / 2 ml ice-cold methanol with light shielding.

LPS and FG

LPS and FG (*Salmonella Typhimurium*) were all obtained from Sigma (St. Louis, MO). LPS was diluted in sterile physiological saline to a final concentration of 0.5 mg/ml. The same lot and dilution of LPS were used for all experiments.

Experiment 1. Effect of Salmonella infection on wheel-running activity in C3H/HeN and C3H/HeJ mice.

C3H/HeN (n=16) and C3H/HeJ (n=16) mice were randomly assigned to one of two groups (n= 8 per each group): PBS (200 μl per mice as vehicle) or live *Salmonella* (Live, 1×10^6 CFU/kg) administered i.p. under light isoflurane anesthesia. Wheel-running activity in both groups of mice was examined by observing their running performance in a cage-adjacent wheel for 24 hr after injection. The experimental procedure was started between 12:30 and 13:00 hours to reduce the variability associated with diurnal rhythms (24,45).

Experiment 2. Effect of HK Salmonella on wheel-running activity.

Male C3H/HeN (n=24) and C3H/HeJ (n=24) mice were randomly assigned to one of three groups (n= 8 per each group): sterile phosphate-buffer saline (PBS, 200 μl per mice as vehicle), LPS (0.5 mg/kg) or HK *Salmonella* (0.5 mg/kg). Each mouse was lightly anesthetized with inhalant Isoflurane prior to the i.p. injection. The experimental procedure was conducted between 12:30 and 13:00 hours to reduce the variability associated with diurnal rhythms. Wheel-running activity in all groups was examined by observing their running performance in a cage-adjacent wheel for 24 hr after the injection.

Experiment 3. Effect of HK and live Salmonella on TNF-α production from macrophages in vitro.

Raw 264 cells ($5x10^4$/ well) in 96 well plates were pre-incubated for 24 hr and then were stimulated for 0-12 hr with PBS, HK *Salmonella* (0-1,000 CFU/well) or live *Salmonella* (0-1,000 CFU/ well). After the stimulation, the supernatants were collected and then stored at -80˚C until analysis of TNF-α via ELISA.

Experiments 4 and 5. Effect of flagella on wheel-running activity.

Male C3H/HeJ (n=32) mice were randomly assigned to one of four groups: PBS as vehicle (200 μl, n=9), *Salmonella* without gentamicin treatment (NT, $1x10^6$ CFU/kg, n=11), *Salmonella* with GM treatment (GMT, $1x10^6$ CFU/kg, n=6) and the supernatant of GM-treated *Salmonella* (SG, 25 mg/kg, n=6). Each mouse was lightly anesthetized with inhalant Isoflurane prior to the i.p. injection. The experimental procedure was conducted between 12:30 and 13:00 hours. Voluntary phys-

ical activity was examined by observing running performance in cage-adjacent wheels for 24 hr after i.p. injection. Moreover, we also tested the effects of FG alone on wheel running activity in C3H/HeN mice. Male C3H/HeN (n=15) mice were randomly assigned to one of two groups: PBS as vehicle (200 μl, n=7) or FG (1 mg/kg, n=8). Wheel-running activity in both groups of mice was examined by observing their running performance in cage-adjacent wheels for 24 hour after PBS or FG injections. The experimental procedure was also started between 12:30 and 13:00 hours. In addition, body weight was measured before and 24 hr after their treatments.

ELISA for TNF-α

TNF-α was measured by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (R&D Systems, Minneapolis, MN). The absorbance was measured at 450 nm and was proportional to the concentration of TNF-α in the sample. The minimum detectable dose of mouse TNF-α was typically less than 5.1 pg/ml.

Statistics

Data are expressed as the means ± S.E.M. Statistical analyses were performed using an analysis of variance procedure (ANOVA) by Stat View for Windows version 5.0. Fisher's protected least-significant difference test was used for post hoc analyses. *P* values of <0.05 were considered statistically significant.

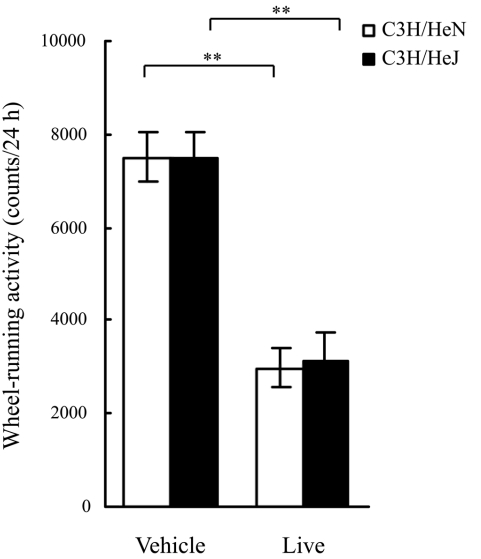

Fig. 1. Effect of live *Salmonella* injection (Live) (1 x 10^6 CFU/kg) on wheel-running activity (24 hr post-injection) in C3H/HeJ) and C3H/HeN mice. The values are expressed the mean ± S.E.M. **p<0.01, n=8 in each group.

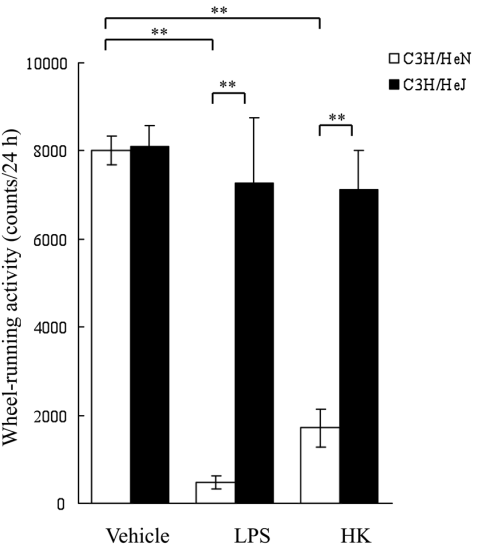

Fig. 2. Effect of LPS (0.5 mg/kg) or HK (0.5 mg/kg) *Salmonella* injection on wheel-running activity (24 hr post-injection) in C3H/HeJ (*tlr* 4 mutation) and C3H/HeN mice. The values are expressed the mean ± S.E.M. **p<0.01, n=8 in each group.

RESULTS

Experiments 1 and 2. Effects of LPS and Salmonella infection on wheel-running activity in C3H/HeN and C3H/HeJ mice.

After treatment with live *Salmonella*, both C3H/HeN (intact *tlr* 4 signaling) and C3H/HeJ (*tlr* 4 mutated) mice exhibited significantly reduced wheel-running activity when compared to vehicle controls ($p<0.01$ and $p<0.01$, respectively, Fig. 1). The level of wheel-running activity was not significantly different between C3H/HeN and C3H/HeJ mice after the injection of live *Salmonella*. These data indicate that *tlr* 4 is not required to induce a reduction in wheel running after *Salmonella* infection. To verify this, and to demonstrate LPS non-responsiveness in our C3H/HeJ mice, we administered HK *Salmonella* (heat-killing breaks apart bacterial cell wall components exposing Lipid A for better binding to *tlr* 4) and LPS to both strains of mice (Fig. 2). In C3H/HeN mice, wheel-running activity in the LPS- and HK-treated groups was greatly reduced when compared to vehicle controls ($p<0.01$ and $p<0.01$, respectively, Fig. 2). Interestingly, while LPS failed to reduce wheel running in *tlr* 4 deficient C3H/HeJ mice as expected, administration of HK *Salmonella* did not affect wheel-running activity in C3H/HeJ mice; indicating that heat-labile structures (not LPS acting through *tlr* 4) may be responsible for reduced wheel running in response to *Salmonella* infection in this strain (Fig. 2).

Experiment 3. Effect of heat-killed and live Salmonella on TNF-α production from macrophages in vitro.

Contribution of pro-inflammatory cytokines, such as TNF-α, to various sickness behaviors in mice has been documented (28). Therefore, to examine the extent to which LPS and live and HK *Salmonella* induced macrophage TNF-α production,

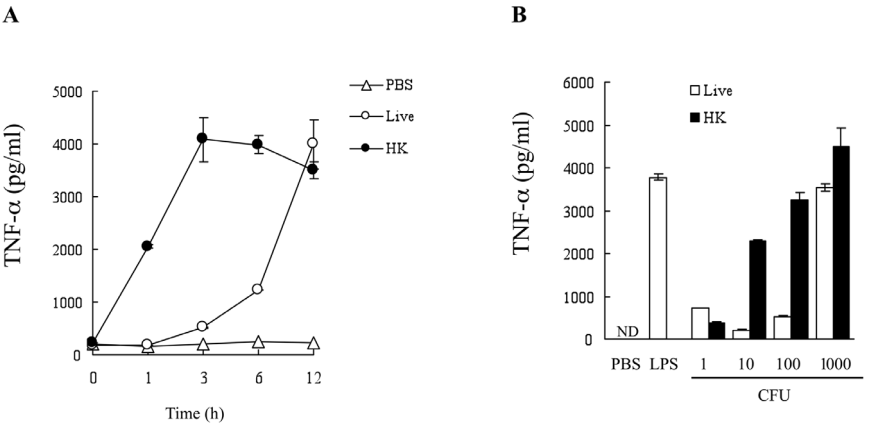

Fig. 3. Effects of HK or live *Salmonella* (Live) challenge on in vitro production of TNF-α in RAW264 macrophages. RAW264 cells (5×10^4 cell/well) were stimulated with HK (100 CFU/ml) or live *Salmonella* (100 CFU/ml) for 0, 1, 3, 6 or 12 hr (Fig. 3A). Cells were also stimulated with 0, 1, 10, 100 or 1,000 CFU/ml of HK, Live, LPS (1 μg/ml) or PBS for 3 hr (Fig. 3B) and TNF-α secretion was determined by ELISA. The values are expressed mean ± S.E.M. of two separate experiments.

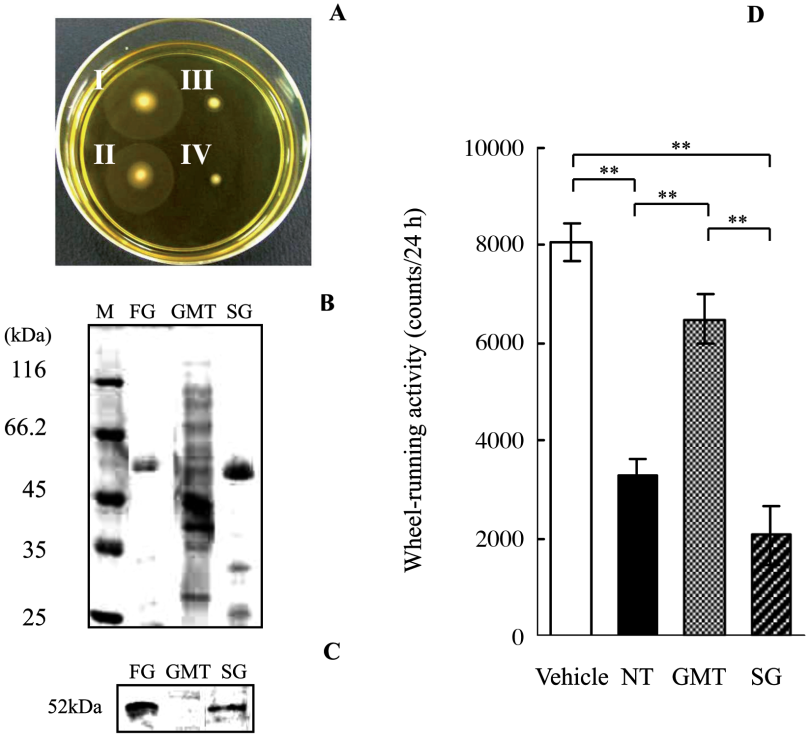

Fig. 4. Effect of gentamicin-treated *Salmonella* administration on wheel-running activity in C3H/HeJ mice. Representative photograph of triplicate individual experiments where *Salmonella* was treated with the following doses of gentamycin (I = 0 mg/ml, II = 1 mg/ml, III = 10 mg/ml, IV = 100 mg/ml and tested for motility in agar (Fig. 4A). Representative (of triplicate gels) SDS-PAGE analysis (stained with Coomassie Blue for 10 min) of gentamicin-treated *Salmonella* (GMT, 20 mg) and the supernatant of gentamicin-treated *Salmonella* (SG, 25 mg) after centrifugation (Fig. 4B). M and FG represent the molecular weight marker and a positive flagellin (25 mg, Sigma) control, respectively. Representative (of 3 separate blots) Western blot analysis of flagellin in GMT and SG (Fig. 4C). Voluntary wheel-running activity in C3H/HeJ mice for 24 hr after i.p. injection with PBS vehicle (200 μl, n=9), *Salmonella* without gentamicin treatment (NT, $1x10^6$ CFU/kg, n=11), GMT ($1x10^6$ CFU/kg, n=6) or SG (25 mg/kg n=6). The values are expressed as mean ± S.E.M. **p<0.01 respectively (Fig. 4D).

we performed an *in vitro* experiment using RAW 264 macrophages. TNF-α production increased after incubation of macrophages with both live and HK *Salmonella* (Fig. 3A), but was more rapid in the HK condition peaking at 3 hr post vs. 12 hr post with live *Salmonella*. In addition, RAW 264 cells were cultured with HK *Salmonella* (0-1,000 CFU/ml) and live *Salmonella* (0-1,000 CFU/ml) for 6 hr (Fig. 3B). At low the low dose (1 CFU/ml), live *Salmonella* , TNF-α production was not different from HK *Salmonella*. However, at higher doses (10-1,000 CFU/ml) HK *Salmonella* (10 and 100 CFU/ml) induced greater TNF-α produc-

tion when compared to live *Salmonella* indicating that HK treatment increases the potency of the proinflammatory effect of *Salmonella*.

Experiments 4 and 5. Effect of flagellin on wheel running activity.
GM (a bacterial protein synthesis inhibitor) treatment dose-dependently reduced *Salmonella* motility (Fig. 4A). SDS-PAGE analysis (Fig. 4B) and western blot analysis (Fig. 4C) confirmed that GM treatment (GMT) reduced FG in *Salmonella*, but not in the supernate (SG) of *Salmonella* cultures. Interestingly, untreated *Salmonella* (NT) and SG significantly reduced wheel running activity 24 hr post administration ($p<0.01$), whereas GMT *Salmonella* did not (Fig. 4D), indicating that FG plays an important role in the *Salmonella*-induced attenuation of wheel-running behavior. Furthermore, both wheel-running activity and body weight were significantly reduced in mice after intravenous FG injection ($p<0.01$, Fig. 5A and $p<0.01$, Fig.5B, respectively).

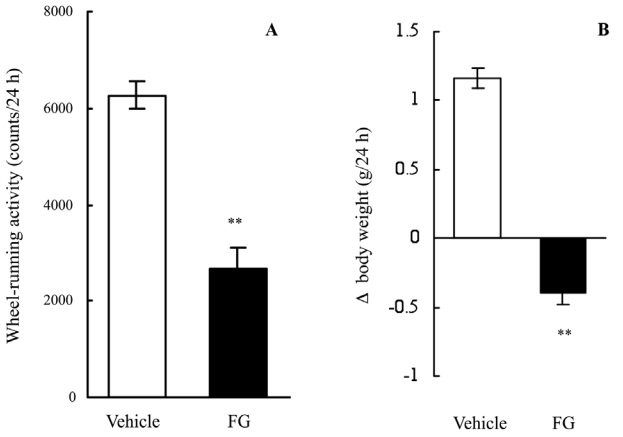

Fig. 5. Wheel-running activity (Fig.5A) and loss of body weight (24 hr post) (Fg.5B) in C3H/HeN mice after flagellin (FG, 1 mg/kg, intravenous injection of 200 μl) injection. The values are expressed as mean ± S.E.M. **p<0.01 v.s Vehicle, respectively. n=7-8 in each group.

DISCUSSION

Systemic bacterial infection results in a vigorous pro-inflammatory cytokine response and various sickness-related behaviors including reduced food intake and lethargy (20,25). Because of the widespread use of LPS as a model for gram-negative bacterially-induced physiological and behavioral changes (5), little attention has been paid to other bacterial structures that could result in pro-inflammatory responses and altered sickness behavior. Accordingly, we hypothesized that *Salmonella* FG, which binds to TLR 5 on host cells (1), also has the ability to promote inflammation and sickness behaviors. Our results clearly indicate that, along with LPS, FG contributes to the reduction in wheel-running activity after *Salmonella* infection.

We demonstrated that *Salmonella* infection attenuated voluntary wheel-running in C3H/HeJ mice. This is significant because this strain of mouse exhibits a point mutation in TLR 4 (31), rendering it incapable of responding to LPS with altered behavior (5). However, the strain still possesses the ability to recognize

bacteria ligands via TLR2, TLR5, and TLR9. LPS activation of TLR4 triggers the biosynthesis of diverse mediators of inflammation and activates the production of costimulatory molecules required for the adaptive physical behavior (1). Indeed, many previous studies suggest that LPS, which is made up of an outer monolayer on the outer membranes of most gram-negative bacteria (33), induces reduction of physical activity (12,17,19,45). Therefore, data from this C3H/HeJ experiment suggest that bacterial components other than LPS must have been responsible for reduced wheel-running behavior. We also subjected both strains of mice to HK *Salmonella*. Interestingly, heat-killing increased the suppressive effect of *Salmonella* (when compared to administration of viable *Salmonella*) on wheel-running activity in C3H/HeN (TLR 4 intact) mice, while having no effect on running behavior in C3H/HeJ mice. The former result is consistent with our idea that heat denaturation, which induces rapid and extensive killing of bacteria, induces release of bacterial cell wall components including LPS (21). Indeed, Vazquez-Torres et al. (44) reported that HK *Salmonella* treatment increased INF-γ staining of $CD4^+$ T cells in C3H/HeN mice, but the adaptive cellular immune responses to HK *Salmonella* were attenuated in C3H/HeJ mice. Moreover, it is also known that macrophages respond better to nonmotile, killed bacteria than to living or motile bacteria (36,37). Our results demonstrated that induction of a decrease in physical activity after HK *Salmonella* injection in C3H/HeN mice most probably occurred by the LPS/TLR4 signaling pathway initiating intercellular messengers and activating NF-κB (2).

Our latter result, demonstrating no wheel activity-reducing effect of HK *Salmonella* when compared to live *Salmonella* in C3H/HeJ mice, indicated that the structure responsible for reduced wheel-running in this strain is heat-labile. Along these lines, it has been demonstrated that heat killing of *Salmonella*, while increasing the binding efficiency of LPS, destroys other components of the bacterial wall including flagella (41,43) and the type III secretion system (4).

Our data indicating that HK treatment of *Salmonella* abrogated the reduction in wheel-running induced by *Salmonella* led us to hypothesize that the difference in wheel-running activity between live *Salmonella* and HK *Salmonella* might be due to differences in the magnitude of the inflammatory response. Therefore, we measured TNF-α production from the macrophage cell line RAW 264 in response to HK or live *Salmonella*. Contrary to our hypothesis, HK *Salmonella* treatment led to an earlier rise in TNF-α production than to live *Salmonella*. Moreover, RAW 264 cells were more sensitive to low doses of HK *Salmonella* when compared to live *Salmonella*. This is consistent with the effect of heat-killing on LPS binding efficiency.

The results of the experiments discussed above led us to investigate other bacterial structures that might contribute to *Salmonella*-induced reductions in wheel-running behavior. Bacteria of the *Salmonella* family produce a number of specialized effecter proteins that can modify host cell signaling (14) and potentially explain the LPS-independent effects seen in our studies. Indeed, antibodies directed against flagella prevent bacterial motility and pathogenesis in mouse models (8,16,38). In addition, it has been reported that live *Salmonella* stimulates early release of TNF-α from RAW cells without TLR4 (37). *Salmonella* has several PAMPs, such as FG, PGN and DNA (1). FG is the major structural protein of flagella expressed in most gram-negative bacteria (16). Recent reports indicate

that flagella elicit host immune responses and that the responsible component is the filament protein FG which acts by binding to TLR5 (40). Indeed, intravenous FG causes activation of the MAPK, SAPK and IKK signaling pathways, and NF-κB activation (42), inducing a systemic inflammatory response (e.g. IL-8, IL-6 and TNF-α) in mice (9,10) and in vitro (46).

In this study, we used gentamycin (GM) an antibiotic bacterial protein synthesis inhibitor, to render *Salmonella* immotile. GM-treated *Salmonella* did not attenuate wheel-running activity in C3H/HeJ mice, whereas the supernate of GM-treated *Salmonella* did result in a significant reduction in wheel-running. SDS PAGE and western blot analysis revealed loss of FG in GM-treated *Salmonella*, but not in GM-treated supernates, indicating that FG may be involved in the reduction of wheel-running induced by *Salmonella* treatment in C3H/HeJ mice. Moreover, FG also reduced wheel-running activity in normal mice. These data implicate FG, acting through TLR 5, as a moderator of reduced wheel-running behavior in mice. In contrast, we did not observe any reduction in wheel-running activity when mice were administered either PGN or CpGDNA (data not shown).

The mRNA expression of TNF-α in response to FG was lower than that in response to LPS (29), and the increase in cytokines (e.g. TNF-α and IL-6) and nitric oxide (NO) produced by FG was also lower than that of LPS (22). In this study, however, a significant reduction in wheel-running activity was observed after the injection of live *Salmonella*, the flagella-rich supernatant of *Salmonella* and purified FG. Although we were unable to answer the question of what is the direct inducer of FG-induced sickness behavior, recent studies have reported that FG induces hypotension (9), severe liver damage (22) and upregulation of IL-8 (41), which is a chemotactic factor and activator of neutorophils, basophils and T cells (26) and is involved in the early host response to pathogens (11,18). Clarification of what proteins dictate the FG/TLR5 signaling pathway that leads to the reduction of physical activity will be required in the future.

In summary, our findings provide evidence that FG expressed on the surface of the gram-negative bacterium *Salmonella* attenuates the reduction of voluntary physical activity in mice.

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