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Systemic LPS induces spinal inflammatory gene expression and impairs phrenic long-term facilitation following acute intermittent hypoxia

A. G. Huxtable, S. M. C. Smith, S. Vinit, J. J. Watters, and G. S. Mitchell

Department of Comparative Biosciences, University of Wisconsin, Madison, Wisconsin

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Huxtable AG, Smith SM, Vinit S, Watters JJ, Mitchell GS. Systemic LPS induces spinal inflammatory gene expression and impairs phrenic long-term facilitation following acute intermittent hypoxia. *J Appl Physiol* 114: 879–887, 2013. First published January 17, 2013; doi:10.1152/japplphysiol.01347.2012.—Although systemic inflammation occurs in most pathological conditions that challenge the neural control of breathing, little is known concerning the impact of inflammation on respiratory motor plasticity. Here, we tested the hypothesis that low-grade systemic inflammation induced by lipopolysaccharide (LPS, 100 μg/kg ip; 3 and 24 h postinjection) elicits spinal inflammatory gene expression and attenuates a form of spinal, respiratory motor plasticity: phrenic long-term facilitation (pLTF) induced by acute intermittent hypoxia (AIH; 3, 5 min hypoxic episodes, 5 min intervals). pLTF was abolished 3 h (vehicle control: 67.1 ± 27.9% baseline; LPS: 3.7 ± 4.2%) and 24 h post-LPS injection (vehicle: 58.3 ± 17.1% baseline; LPS: 3.5 ± 4.3%). Pretreatment with the nonsteroidal anti-inflammatory drug ketoprofen (12.5 mg/kg ip) restored pLTF 24 h post-LPS (55.1 ± 12.3%). LPS increased inflammatory gene expression in the spleen and cervical spinal cord (homogenates and isolated microglia) 3 h postinjection; however, all molecules assessed had returned to baseline by 24 h postinjection. At 3 h post-LPS, cervical spinal iNOS and COX-2 mRNA were differentially increased in microglia and homogenates, suggesting differential contributions from spinal cells. Thus LPS-induced systemic inflammation impairs AIH-induced pLTF, even after measured inflammatory genes returned to normal. Since ketoprofen restores pLTF even without detectable inflammatory gene expression, "downstream" inflammatory molecules most likely impair pLTF. These findings have important implications for many disease states where acute systemic inflammation may undermine the capacity for compensatory respiratory plasticity.

spinal cord; inflammation; plasticity; respiratory motor neuron; microglia; inflammatory genes

SYSTEMIC INFLAMMATION OCCURS in most clinical disorders that challenge the neural control of breathing, including chronic obstructive lung disease (6, 60, 70); traumatic, ischemic, and degenerative neural disorders (e.g., spinal injury, motor neuron disease) (1, 46, 63); and sleep-disordered breathing (7, 24, 33). Although inflammation affects many neural functions (18), the impact of inflammation on the neural system controlling breathing has seldom been studied. Because of the importance of ventilatory control in many clinical disorders, it is essential to understand connections between inflammation and essential processes of ventilatory control, including rhythm generation, chemoreception, and plasticity (19, 41). In this study we focus on the impact of inflammation on an important model of respiratory plasticity, phrenic long-term facilitation following acute intermittent hypoxia (AIH).

Address for reprint requests and other correspondence: G. Mitchell, Department of Comparative Biosciences, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI, 53706 (e-mail: mitchell@svm.vetmed.wisc.edu).

Neuroinflammation involves complex spatial and temporal patterns of inflammatory gene expression (reviewed in 40). In the central nervous system (CNS), resident microglia are major contributors to the inflammatory response and are activated by many pathological stimuli. Upon activation, microglia change shape from stellate, ramified cells to an amoeboid shape (35) and begin to release proinflammatory and anti-inflammatory molecules (e.g., cytokines, nitric oxide, prostaglandins, and growth/trophic factors) (25). The influence of microglial inflammation on respiratory plasticity is the focus of the present study.

Systemic administration of the bacterial endotoxin lipopolysaccharide (LPS) decreases baseline ventilation and ventilatory responses to chemoreceptor stimulation in unanesthetized rats (29). These effects are reversed by pretreatment with the nonsteroidal anti-inflammatory drug ketoprofen, (29). LPS is a toll-like receptor ligand frequently used to study systemic inflammation (48). Although LPS does not cross the blood-brain barrier (49, 58), it elicits CNS inflammation via systemic release of cytokines (which do cross the blood-brain barrier) or vagal transmission (9, 23, 36, 39, 52, 55). Systemic LPS administration impairs hippocampal synaptic plasticity, as well as learning and memory (18). Similarly, systemic LPS impairs an important model of spinal respiratory plasticity: AIH-induced phrenic long-term facilitation (pLTF) (29, 67). In the earlier study of Vinit et al. (67), *1*) spinal inflammation was not confirmed, *2*) only high LPS doses (3 mg/kg) and a short time interval (3 h) were studied, and *3*) causality between LPS-induced inflammation and pLTF impairment was not investigated. Here, we extend the results of Vinit et al. (67) by testing the hypotheses that *1*) low LPS doses impair pLTF at longer intervals postadministration (24 h), *2*) systemic LPS increases inflammatory gene expression in isolated microglia and homogenates from the cervical spinal cord (a critical region for pLTF), and *3*) the nonsteroidal anti-inflammatory drug ketoprofen restores pLTF following LPS administration. Our results support the hypothesis that systemic inflammation impairs spinal respiratory plasticity and demonstrate that systemic inflammation associated with many clinical disorders is of considerable relevance to the central neural control of breathing.

METHODS

All experiments were approved by the Animal Care and Use Committee in the School of Veterinary Medicine, University of Wisconsin, and conformed to policies laid out by the National Institutes of Health in the *Guide for the Care and Use of Laboratory Animals*. Experiments were performed on 3- to 4-mo-old Harlan male Sprague-Dawley rats (colony 218a). Rats were housed under standard conditions, with a 12:12-h light/dark cycle with food and water ad libitum.

Drugs and Materials

LPS (*E. coli* 0111:B4), (*S*)-(+)-ketoprofen, and Tri Reagent were purchased from Sigma Chemical (St. Louis, MO). M-MLV Reverse Transcriptase was purchased from Invitrogen (Carlsbad, CA). Oligo(dT), Random hexamer, and RNAsin were purchased from Promega (Madison, WI). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Carlsbad, CA).

Experimental Groups

To investigate the effects of systemic inflammation, rats received an intraperitoneal (ip) injection of LPS (100 μg/kg) or vehicle (saline) 3 or 24 h prior to beginning an experiment. Rats were either used for electrophysiological experiments to study AIH-induced pLTF or for measurements of inflammatory gene expression. In the latter groups, intra-aortic saline perfusions were used to remove circulating myeloid cells before cervical spinal tissues were harvested. Prior to perfusions, the spleen was harvested for analysis.

In 24 h LPS or 24 h vehicle rats, the nonsteroidal anti-inflammatory drug ketoprofen (12.5 mg/kg ip) or vehicle (50% ethanol in saline) was injected 3 h prior to a pLTF experiment. Ketoprofen is a potent inhibitor of inflammatory activities since it directly inhibits cyclooxygenase (31, 65, 68) and the transcription factor nuclear factor kappa B (NF-κB) (65), a key regulator of multiple inflammatory genes (42, 51).

In specific, rats used for electrophysiology experiments after 3 h of LPS fell into one of three groups: *1*) time control (includes both vehicle and LPS injected), *2*) vehicle + AIH, or *3*) LPS (3 h) + AIH. A separate set of rats was used to examine the effects of 24 h of LPS and fell into one of five groups: *1*) time control (includes vehicle, LPS, ketoprofen, or LPS + ketoprofen injected); *2*) LPS (24 h), ketoprofen + AIH; *3*) LPS (24 h), vehicle + AIH; *4*) vehicle (24 h), ketoprofen + AIH; and *5*) vehicle + AIH.

For gene expression, rats were in comparable groups as those above for the electrophysiology. Rats were in either LPS (3 h), vehicle (3 h), LPS (24 h) + ketoprofen, LPS (24 h) + vehicle, vehicle (24 h) + ketoprofen, or vehicle groups. The rats used for gene expression analysis were not given AIH, neglecting the need for a time control group.

Electrophysiological Experiments

The protocol used in electrophysiological experiments has been described in detail previously (2, 5). In brief, rats were anesthetized with isoflurane, tracheotomized, and pump ventilated (Small Animal Ventilator 683, Harvard Apparatus, Holliston, MA). Rats were maintained with isoflurane for the remainder of the surgical preparation; after surgical preparations were complete, the rats were slowly converted to urethane anesthesia (1.8 g/kg iv, Sigma-Aldrich). During the 1-h stabilization period after conversion to urethane, pancuronium bromide (1 mg iv), was given to paralyze the rats. The rats were tracheotomized and ventilated. Anesthetic level was assessed throughout experiments by monitoring blood pressure and phrenic nerve responses to toe pinch. Approximately 1 h after beginning surgical procedures, an intravenous infusion of 1.5–2 ml/h began with a solution consisting of Hetastarch (0.3%) and sodium bicarbonate (0.84%) in lactated Ringer's. The infusion rate was adjusted to maintain blood volume, pressure, and acid-base balance.

Surgical preparation. Both vagi were isolated and cut, and a catheter was inserted into the right femoral artery to enable blood pressure measurements and arterial blood samples. Blood samples were analyzed for P_{O_2}, P_{CO_2}, pH, and base excess using a blood gas analyzer (ABL 800, Radiometer, Copenhagen, Denmark). Blood samples (62.5 μl in heparinized plastic catheter) were drawn before (baseline), during the first hypoxic episode, and 15, 30 and 60 min post-AIH. A rectal temperature probe was used to monitor and regulate body temperature throughout an experiment.

The left phrenic nerve was isolated with a dorsal approach, cut caudally, desheathed, and placed on a bipolar silver recording electrode submerged in mineral oil. Nerve activity was amplified (gain X10K), bandpass filtered (300 Hz to 20 kHz) (A-M Systems, Carlsberg, WA), and integrated (absolute value, Powerlabs 830, AD Instruments, Colorado Springs, CO, time constant 100 ms). The signal was digitized, recorded, and analyzed using Powerlabs 830 (version 7.2.2, AD Instruments).

Protocol. Baseline nerve activity was established with $FIO_2 \sim 0.56$ ($PaO_2 > 300$ mmHg) and CO_2 added to the inspired gas (balance nitrogen). The CO_2 apneic threshold was determined by progressively lowering inspired CO_2 until phrenic nerve activity ceased. Inspired CO_2 was slowly increased until phrenic nerve activity resumed (recruitment threshold). End-tidal CO_2 was then set 3 mmHg above the recruitment threshold to establish baseline nerve activity. End-tidal CO_2 was monitored and maintained throughout an experiment using a flow-through capnograph (Respironics, Andover, MA).

Once phrenic nerve activity was stable, an arterial blood sample was taken to establish baseline conditions; these conditions were maintained throughout the experiment. After baseline conditions, an AIH protocol began consisting of three hypoxic episodes (5 min duration, 10.5% O_2), separated by 5 min of normoxia. Blood samples were taken during the first hypoxic episode and 15, 30, and 60 min post-AIH. Data were included in analysis only if they complied with the following criteria: *1*) PaO_2 during baseline and post-AIH was >180 mmHg; *2*) PaO_2 during hypoxic episodes was between 35 and 45 mmHg; *3*) $PaCO_2$ remained within 1.5 mmHg of baseline throughout the post-AIH period. Phrenic nerve amplitude and frequency were evaluated for 1 min before each blood sample. Upon completion of the experiment, rats were euthanized with an overdose of urethane.

Inflammatory Gene Expression

Sample preparation. Tissue from cervical spinal cords was homogenized and used for quantitative PCR analysis (hereafter referred to as "homogenates"). Cervical spinal tissue was also used to isolate microglia by using antibodies for the microglial marker $CD11b^+$ conjugated to magnetic beads, as previously reported (15). To isolate microglia, these labeled cells were passed through MS columns according to a modified Miltenyi MACS protocol (43). The average purity of cells with microglia-like characteristics was >95% as determined by flow cytometry FSC/SSC scatter analysis and $CD11b^+/CD45^{low}$ staining (43). These results are consistent with previous findings (15, 56). From here on, $CD11b^+$ cells isolated with this method are referred to as "microglia."

Reverse transcription. Total RNA was isolated from cervical spinal cord microglia or homogenates using the TRI-reagent according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase and an oligo(dT)/random hexamer cocktail. The cDNA was then used for quantitative RT-PCR using SYBR Green PCR Master Mix.

Quantitative PCR. Amplified cDNA was measured by fluorescence in real time using the StepOnePlus Real-Time PCR System (Applied Biosystems). The following primer sequences were used for quantitative PCR: iNOS, 5′-AGG GAG TGT TGT TCC AGG TG and 5′-TCT GCA GGA TGT CTT GAA CG; COX-2, 5′-TGT TCC AAC CCA TGT CAA AA and 5′-CGT AGA ATC CAG TCC GGG TA; IL-1β, 5′-CTG CAG ATG CAA TGG AAA GA and 5′-TTG CTT CCA AGG CAG ACT TT; TNF-α, 5′-TCC ATG GCC CAG ACC CTC ACA C and 5′-TCC GCT TGG TGG TTT GCT ACG; IL-6, 5′-GTG GCT AAG GAC CAA GAC CA and 5′-GGT TTG CCG AGT AGA CCT CA; and 18s, 5′-CGG GTG CTC TTA GCT GAG TGT CCC G and 5′-CTC GGG CCT GCT TTG AAC AC.

All primers were designed to span introns whenever possible. Primer specificity was assessed through NCBI BLAST analysis prior to use, and all dissociation curves had a single peak with an observed melting temperature consistent with the intended amplicon sequences.

Primer efficiency was calculated through the use of serial dilutions and construction of a standard curve.

Data Analysis

Electrophysiology. Peak amplitude and frequency (bursts/min) of integrated phrenic nerve activity were averaged for 30 bursts at each recorded point. Changes in phrenic nerve burst amplitude were normalized to baseline values (i.e., percent change from baseline), and burst frequency was reported as a change from baseline frequency (bursts/min). In this study, physiological variables and phrenic amplitude are reported for baseline, during the short-term hypoxic response, and the 60 min time point only.

Statistical comparisons for the hypoxic responses were made from data at *minute 2* of the 5 min during the first hypoxic episode using a *t*-test (3 h LPS data) or one-way ANOVAs on ranks (24 h LPS data). Owing to the addition of two ketoprofen groups, an ANOVA on ranks was used for the 24 LPS hypoxic data to permit appropriate statistical comparisons. Nonparametric analyses were used when data failed normality or equal variance.

Statistical comparisons for changes in phrenic burst amplitude after AIH were made using a repeated-measures two-way ANOVA with Tukey post hoc test to identify individual differences (Sigma Stat version 11, Systat Software, San Jose, CA). Differences were considered significant if $P < 0.05$. All values are expressed as means ± SE.

Gene expression. Gene expression data were analyzed based on a relative standard curve method, as specified by Applied Biosystems. In brief, all samples were run in duplicate, averaged, and interpolated onto previously run standard curves for each primer set to account for differences in primer efficiency. Values were then normalized to 18S for each sample and expressed relative to vehicle controls for each gene, reflecting the fold change for each gene. If the normalized gene expression data for an individual sample is greater than 2 standard deviations from the mean, the sample was excluded as an outlier. Statistical analysis for TNFα and IL-1β were run on the fold change data. Statistical analysis on iNOS, COX-2, and IL-6 failed equal variance and/or normality tests; therefore data were transformed logarithmically before statistical analysis, but data are still reported as fold changes (see Fig. 4). Statistical significance was determined for each inflammatory gene examined in the spleen by a one-way ANOVA with Tukey post hoc test for individual comparisons. For cervical spinal data, statistical significance was determined using a two-way ANOVA with Tukey post hoc test (Sigma Stat version 11, Systat Software, San Jose, CA). Differences were considered significant if $P < 0.05$. All values are expressed as means ± SE.

RESULTS

Impaired pLTF 3 h post-LPS. Acute LPS (3 h; 100 μg/kg ip) had only minor effects on physiological variables measured (Table 1). Rats treated with LPS had no significant differences in temperature, $PaCO_2$, or pH within or between groups. However, LPS rats had significantly lower PaO_2 and mean arterial pressure (MAP) after AIH vs. time controls but were not different from their respective baseline values. LPS-treated rats were not significantly different from vehicle-treated rats in any variable examined. In LPS- and vehicle-treated rats subjected to AIH, PaO_2 significantly decreased during hypoxic episodes, with a concurrent decrease in MAP; no similar changes were noted in time control rats without AIH.

LPS did not reduce phrenic nerve burst amplitude during hypoxia, nor did it change frequency at any time during experiments. The short-term hypoxic phrenic response (the immediate increase in phrenic nerve amplitude in response to decreased oxygen) was not significantly affected by LPS (vehicle: 147 ± 46% baseline; LPS-treated: 83 ± 15%; $P = 0.261$; *t*-test, Fig. 1*B*). Baseline phrenic burst frequency was 39 ± 2 bursts/min in the vehicle group, 43 ± 3 bursts/min in the LPS group, and 43 ± 2 bursts/min in the time control group. Similarly, there were no significant changes in frequency responses during hypoxia (vehicle: 10.0 ± 2.0 burst/min; LPS: 12.8 ± 2.8 bursts/min; $P = 0.453$; *t*-test) or following AIH (vehicle: 1.0 ± 2.0 bursts/min at 60 min post-AIH, LPS: 5.5 ± 2.4 bursts/min; time control: -1.9 ± 1.6 bursts/min; data not shown).

Acute LPS significantly diminished pLTF magnitude vs. vehicle controls (vehicle: 67.1 ± 27.9% baseline, $n = 5$; LPS: 3.7 ± 4.2% baseline, $n = 5$; Fig. 1*C*; $P < 0.001$; repeated-measures two-way ANOVA, Tukey post hoc test). There was no significant difference between LPS-treated and time control rats (5.1 ± 4.3%, $n = 5$). Thus this low LPS dose (100 μg/kg) abolishes pLTF shortly after administration (3 h), similar to previous findings with a higher LPS dose (3 mg/kg) (67). Neither this dose (3 mg/kg) nor that of Vinit et al. (67) caused overt sickness behaviors in rats (increased temperature, lethargy). Higher LPS doses are used to simulate sepsis, and overt sickness behaviors can be observed (37, 45).

pLTF remains impaired 24 h post-LPS. Because inflammation initiates complex signaling cascades that can persist well beyond 3 h, we examined pLTF 24 h post-LPS injection. Since no significant differences were found in pLTF among the various time control groups (vehicle, LPS, ketoprofen, or LPS + ketoprofen), we combined these groups for further analysis. Further, rats treated with LPS + ketoprofen vehicle were

Table 1. *Physiological parameters for Sprague-Dawley rats during electrophysiological experiments after 3 h of LPS*

		Temperature		PaO_2		$PaCO_2$		pH		MAP	
Time	Treatment Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Baseline	3 h Time control	37.5	0.1	351.8	6.7	45.8	1.3	7.362	0.008	132.5	3.9
	3 h Vehicle	37.7	0.1	331.25	14.1	47.6	1.4	7.356	0.006	128.2	2.8
	3 h LPS	37.5	0.3	351.2	3.7	47.0	0.7	7.353	0.013	125.4	3.9
Hx	3 h Time control	37.5	0.1	349.4	7.4[a]	45.8	1.8	7.360	0.008	137.6	3.8[d]
	3 h Vehicle	37.4	0.1	38.5	2.7[c]	48.5	1.0	7.338	0.008	87.3	5.1[c]
	3 h LPS	37.3	0.2	41.5	2.6[c]	48.9	1.0	7.336	0.010	75.6	9.1[c]
60 min	3 h Time control	37.7	0.02	347.4	3.7	45.4	1.7	7.375	0.008	130.1	2.8[e]
	3 h Vehicle	37.8	0.1	312.8	20.4[bb]	48.4	1.3	7.365	0.015	117.6	6.2
	3 h LPS	37.8	0.1	317.6	6.9[b]	47.1	0.9	7.364	0.022	111.9	3.7

Temperatures are in °C; PaO_2, $PaCO_2$, and MAP are in mmHg. There were no significant differences within or between groups in temperature, $PaCO_2$, or pH. [a]$P < 0.001$, significant difference from all other hypoxia (Hx) groups. [b]$P < 0.05$, significant difference from time control within 60 min. [bb]$P < 0.01$, significant difference from time control within 60 min. [c]$P < 0.001$, significant difference from all other within group. [d]$P < 0.001$, significant difference from all other groups in Hx. [e]$P < 0.05$, significant difference from LPS.

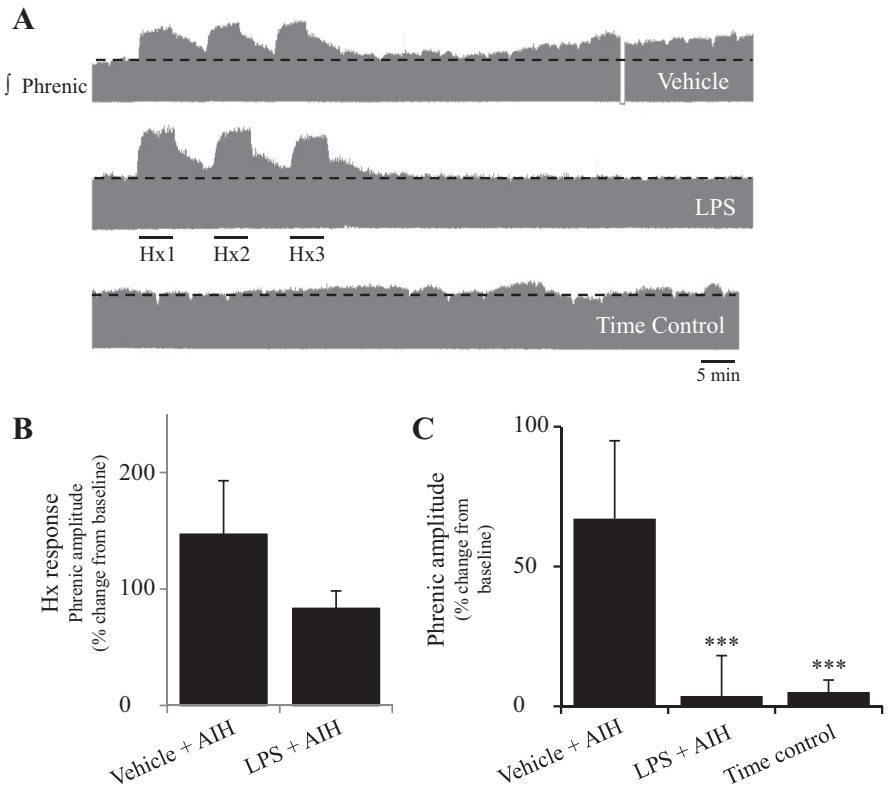

Fig. 1. Systemic inflammation (3 h) induced by LPS (100 μg/kg ip) significantly reduced acute intermittent hypoxia (AIH)-induced phrenic long-term facilitation (pLTF). *A*: representative integrated phrenic neurograms from anesthetized rats during the AIH (3 × 5 min hypoxia: Hx1, Hx2, Hx3) protocol for a vehicle-injected (saline, top trace), or LPS-injected (middle trace), or time control (no AIH, bottom trace) rats. Black dashed line indicates baseline phrenic amplitude in each trace. Development of pLTF is evident as a progressive increase in phrenic nerve amplitude over 60 min in the vehicle-injected animal. *B*: no change in the short-term hypoxia response was evident. *C*: group data for vehicle-injected AIH ($n = 5$), LPS-injected AIH ($n = 5$), and time control ($n = 5$) demonstrating a significant reduction in the magnitude of pLTF 60 min post-AIH in LPS treated and time control rats (***$P < 0.001$ repeated-measures two-way ANOVA, Tukey post hoc test).

combined with the LPS alone group since ketoprofen vehicle had no significant effects and are referred to as 24 h LPS.

Similar to 3 h post-LPS, only minor differences were observed in physiological variables 24 h post-LPS (Table 2). There were no significant differences within or between groups for temperature, $PaCO_2$, or pH. LPS-injected rats had higher MAP levels vs. vehicle-injected rats at baseline, during hypoxia, and after AIH, although neither group differed significantly from time control rats at baseline or post-AIH. LPS-treated rats also had higher MAP compared with rats treated with ketoprofen (without LPS) post-AIH. All treatment groups showed a significant drop in PaO_2 and MAP during hypoxia, as expected.

The short-term hypoxic phrenic response was not significantly altered 24 h post-LPS (Fig. 2, *A* and *B*). There were no significant differences in phrenic nerve burst amplitude during

Table 2. *Physiological parameters for Sprague-Dawley rats during electrophysiological experiments 24 h after LPS*

		Temperature		PaO_2		$PaCO_2$		pH		MAP	
Time	Treatment Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Baseline	24 h Time control	37.5	0.2	335.7	7.4	45.0	0.9	7.334	0.011	130.6	6.0
	24 h Vehicle	37.4	0.3	327.4	12.2	47.4	1.6	7.351	0.014	115.5	8.5[d]
	Ketoprofen	37.7	0.1	328.4	11.4	44.0	1.1	7.365	0.015	130.6	4.0
	24 h LPS	37.6	0.2	325	11.3	45.9	0.4	7.334	0.007	149.8	9.8
	24 h LPS + ketoprofen	37.4	0.2	326	8.5	46.4	1.3	7.3165	0.012	143.4	8.9
	24 h LPS + ketoprofen vehicle	37.6	0.2	342.5	7.6	46.3	2.0	7.324	0.022	140.9	10.9
Hx	24 h Time control	37.5	0.2	336.7	6.8[a]	45.7	1.0	7.334	0.012	129.6	6.2[a]
	24 h Vehicle	37.4	0.3	37.9	2.9[b]	47.8	1.3	7.351	0.014	75.3	20.6[c,d]
	Ketoprofen	37.6	0.1	39.6	1.7[b]	45.8	2.0	7.365	0.015	57.7	8.2[b,d]
	24 h LPS	37.6	0.2	39.7	2.1[b]	47.8	0.3	7.334	0.008	111.0	18.0[b]
	24 h LPS + ketoprofen	37.4	0.2	39.3	1.7[b]	47.9	1.6	7.317	0.012	88.1	10.6[b]
	24 h LPS + ketoprofen vehicle	37.4	0.1	42.8	0.7[b]	44.8	2.0	7.324	0.022	82.8	21.7[b]
60 min	24 h Time control	37.4	0.2	334.3	6.3	45.8	0.8	7.372	0.011	126.5	6.2
	24 h Vehicle	37.2	0.3	326.0	9.0	48.8	1.0	7.351	0.017	105.6	7.7[d]
	Ketoprofen	37.7	0.1	322.2	6.1	44.3	1.4	7.380	0.022	112.9	8.3[d]
	24 h LPS	37.6	0.2	319.6	8.0	45.8	0.6	7.361	0.012	145.2	9.7
	24 h LPS + ketoprofen	37.4	0.1	319.2	11.1	46.5	1.4	7.360	0.011	134.2	10.2
	24 h LPS + ketoprofen vehicle	37.6	0.1	310.5	8.9	46.1	1.8	7.375	0.017	133.8	10.1

Temperatures are in °C; PaO_2, $PaCO_2$, and MAP are in mmHg. There were no significant differences in temperature, $PaCO_2$, or pH. [a]$P < 0.001$, significant difference from all other Hx groups. [b]$P < 0.001$, significant difference from other time points within group. [c]$P < 0.05$, significant difference from baseline within group. [d]$P < 0.05$, significant difference from 24 h LPS.

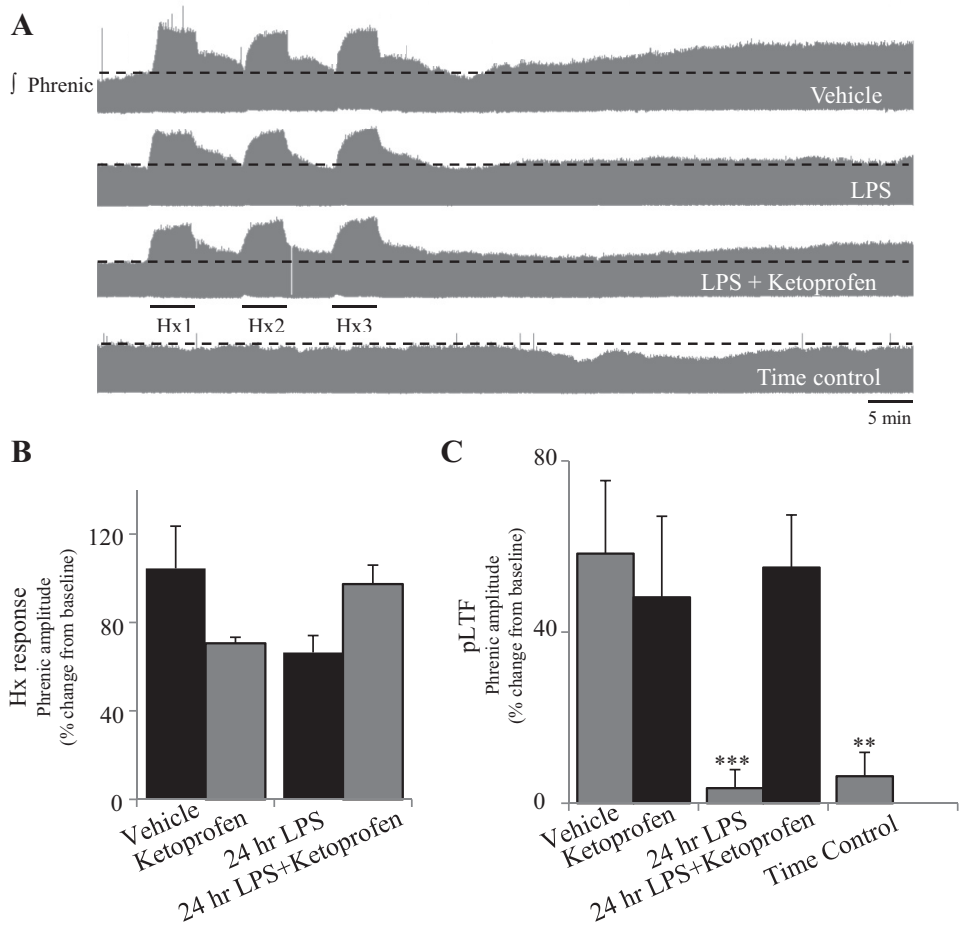

Fig. 2. Systemic inflammation (24 h) induced by LPS (100 μg/kg ip) did not alter the short-term hypoxia response, significantly reduced AIH-induced pLTF, but pLTF was restored with the anti-inflammatory drug ketoprofen (12.5 mg/kg ip, 3 h). *A*: representative integrated phrenic neurograms from anesthetized rats during the AIH (3 × 5 min hypoxia) protocol for vehicle-injected (saline, top trace), LPS-injected (second trace), LPS + ketoprofen-injected (third trace), and time control (no AIH, bottom trace) rats. Black dashed line indicates baseline phrenic amplitude in each trace. Development of pLTF was evident as a progressive increase in phrenic nerve amplitude over 60 min. *B*: no change in the short-term hypoxia response was evident. *C*: group data showing pLTF for vehicle-injected ($n = 5$) and ketoprofen-injected ($n = 6$) rats with AIH, and a reduction in pLTF in rats injected with LPS ($n = 9$). The appearance of pLTF was restored in rats injected with LPS and after treatment with ketoprofen ($n = 6$). There was no increase in phrenic nerve amplitude in time control rats ($n = 15$). (***$P < 0.001$, **$P < 0.01$ indicates significant difference from vehicle, ketoprofen, and 24 h LPS + ketoprofen).

hypoxia in the vehicle group (104 ± 19%;), ketoprofen (71 ± 3%), 24 h LPS (66 ± 8%), or 24 h LPS + ketoprofen (97 ± 9%) ($P > 0.05$, one-way ANOVA on ranks). Baseline phrenic burst frequency was 44 ± 2 bursts/min for time controls, 42 ± 1 bursts/min for 24 h vehicle, 43 ± 2 bursts/min for ketoprofen, 47 ± 2 bursts/min for 24 h LPS, 46 ± 2 bursts/min for 24 h LPS + ketoprofen, and 46 ± 1 bursts/min for 24 h LPS + ketoprofen vehicle. There was a modest effect on phrenic burst frequency at 60 min post-AIH 24 h post-LPS (data not shown). The change in phrenic burst frequency post-AIH in vehicle-treated rats was 5.9 ± 2.3 bursts/min ($n = 5$), whereas the 24 h LPS group was −1.8 ± 1.6 bursts/min ($n = 9$) and time controls were −0.9 ± 1.2 bursts/min ($n = 15$). The change in the vehicle group was significantly different vs. LPS ($P = 0.005$) and time control ($P = 0.010$) groups but not vs. ketoprofen (2.6 ± 2.1 bursts/min, $n = 5$, $P = 0.687$) or 24 h LPS + ketoprofen (0.4 ± 1.4 bursts/min, $n = 6$, $P = 0.149$) groups (repeated-measures two-way ANOVA, Tukey post hoc test).

Impaired pLTF persisted 24 h post-LPS, and this effect was reversed by pretreatment with ketoprofen (Fig. 2*C*). In vehicle-injected rats (58.3 ± 17.1%; $n = 5$) and in rats treated with ketoprofen (48.1 ± 19.0%, $n = 5$), AIH elicited similar pLTF ($P = 0.948$, repeated-measures two-way ANOVA, Tukey post hoc test). pLTF was significantly reduced 24 h post-LPS (3.5 ± 4.3%, $n = 9$, $P < 0.001$ vs. vehicle, $P = 0.004$ vs. ketoprofen) and this effect was reversed by ketoprofen (55.1 ± 12.3%, $n = 6$; $P = 0.999$ vs. vehicle; $P = 0.985$ vs. ketoprofen; $P < 0.001$ vs. LPS) (repeated-measures two-way ANOVA, Tukey post hoc test).

Peripheral inflammatory gene expression. Since LPS does not cross the blood-brain barrier, peripheral LPS-induced inflammation indirectly triggers CNS inflammatory responses (9, 23, 36, 39, 53, 55). Thus we assessed splenic inflammatory gene expression as a marker for systemic inflammation; the same genes were assessed in the cervical spinal cord 3 and 24 h post-LPS. Ketoprofen effects on LPS-induced changes were evaluated only at 24 h post-LPS.

A transient increase in all inflammatory genes examined was evident in the spleen (Fig. 3). Three hours post-LPS ($n = 3$), TNFα (7.5 ± 0.2 fold, $P < 0.001$), iNOS (72.6 ± 12.3 fold, $P < 0.001$), COX-2 (4.4 ± 0.2 fold, $P = 0.013$), IL-1β (4.6 ± 0.1 fold, $P < 0.001$), and IL-6 (19.2 ± 3.5 fold, $P < 0.001$) expressions all significantly increased vs. respective vehicle controls. However, by 24 h post-LPS, mRNA levels for all splenic genes had returned toward baseline values (TNFα: 1.0 ± 0.1 fold, $P = 0.995$; iNOS: 3.4 ± 1.6 fold, $P = 0.083$; COX-2: 1.1 ± 0.1 fold, $P = 0.844$; IL-1β: 1.6 ± 0.4 fold, $P = 0.943$; IL-6: 2.2 ± 0.8 fold, $P = 0.640$). Gene expression 24 h post-LPS was not significantly altered by ketoprofen (TNFα 1.2 ± 0.01 fold, $P = 0.829$; iNOS 1.8 ± 0.6 fold, $P = 0.233$; COX-2 0.8 ± 0.1 fold, $P = 0.981$; IL-1β 1.1 ± 0.1 fold, $P = 0.939$; IL-6 1.1 ± 0.02 fold, $P = 0.956$), despite the ability of ketoprofen to restore pLTF.

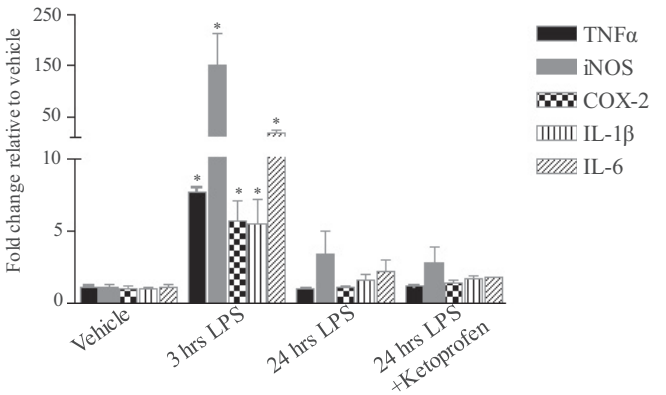

Fig. 3. Systemic inflammation induced by LPS (100 μg/kg ip) caused a transient increase in inflammatory gene expression in the spleen. LPS (3 h) caused a significant increase in all inflammatory genes ($n = 3$) examined compared with the respective vehicle control ($n = 4$) but returned to baseline levels by 24 h ($n = 3$) and was not altered with ketoprofen ($n = 2$) (*$P \leq 0.001$ different from all other treatment groups).

Cervical spinal gene expression (3 h). Inflammatory genes were examined in cervical spinal microglia and homogenates post-LPS (Fig. 4). Only two inflammatory genes exhibited significant increases within microglia or homogenates after LPS: iNOS (microglia 16.7 ± 4.4, $P < 0.001$, $n = 8$; homogenate 8.9 ± 1.9, $P < 0.001$, $n = 8$) and COX-2 (microglia 2.8 ± 0.6, $P = 0.019$, $n = 7$; homogenate 5.1 ± 1.2, $P < 0.001$, $n = 8$) vs. vehicle controls (iNOS: microglia $n = 15$, homogenate $n = 14$; COX-2: microglia $n = 15$, homogenate $n = 15$). There were no significant differences in gene expression between sample type (microglia vs. homogenate) for iNOS or COX-2 (iNOS $P = 0.172$, COX-2 $P = 0.087$). There were no differences in either sample type vs. vehicle controls after LPS ($n = 14$) for IL-1β (microglia 0.7 ± 0.1 fold, $P = 0.562$, $n = 8$; homogenate 0.8 ± 0.3 fold, $P = 0.884$, $n = 7$) or TNFα (microglia 0.9 ± 0.2, $P > 0.05$, $n = 7$; homogenates 1.7 ± 0.3, $P > 0.05$, $n = 7$).

Cervical spinal gene expression (24 h). LPS (24 h) had no significant effects on iNOS (microglia: 2.4 ± 0.8 $P = 0.368$, $n = 7$; homogenates 1.5 ± 0.3 $P = 0.557$, $n = 6$), COX-2 (microglia: 1.0 ± 0.3, $P = 0.978$, $n = 7$; homogenates: 2.1 ± 0.8, $P = 0.473$, $n = 6$), IL-1β (microglia: 0.6 ± 0.2, $P = 0.464$, $n = 7$; homogenates: 0.5 ± 0.2, $P = 0.390$, $n = 5$), or TNFα (microglia: 1.6 ± 0.4, $P > 0.05$, $n = 7$; homogenates 1.7 ± 0.5, $P > 0.05$, $n = 6$) in either sample type vs. vehicle controls (Fig. 4). However, mRNA for iNOS (microglia $P < 0.001$, homogenates $P < 0.001$; Fig. 4*A*) and COX-2 (microglia $P = 0.024$, homogenates $P = 0.013$; Fig. 4*B*) was significantly reduced vs. 3 h LPS within each sample type, suggesting that inflammatory gene expression was returning to baseline values.

Ketoprofen did not significantly alter gene expression in 24 h post-LPS rats ($P > 0.05$) in either sample type for any gene examined (Fig. 4). Nor did ketoprofen alter gene expression vs. vehicle controls for iNOS (microglia: 2.1 ± 0.6, $P = 0.257$, $n = 8$; homogenates: 0.8 ± 0.1, $P = 0.964$, $n = 7$), COX-2 (microglia: 0.9 ± 0.1, $P = 0.892$, $n = 8$; homogenates: 2.2 ± 0.5, $P = 0.066$, $n = 7$), IL-1β (microglia: 0.5 ± 0.1, $P = 0.302$, $n = 8$; homogenates: 0.4 ± 0.1, $P = 0.172$, $n = 5$), or TNFα (microglia: 1.9 ± 0.8, $P > 0.05$, $n = 8$; homogenates: 2.2 ± 1.1, $P > 0.05$, $n = 7$).

Ketoprofen pretreatment did, however, highlight differences between sample types (microglia vs. homogenate) (Fig. 4).

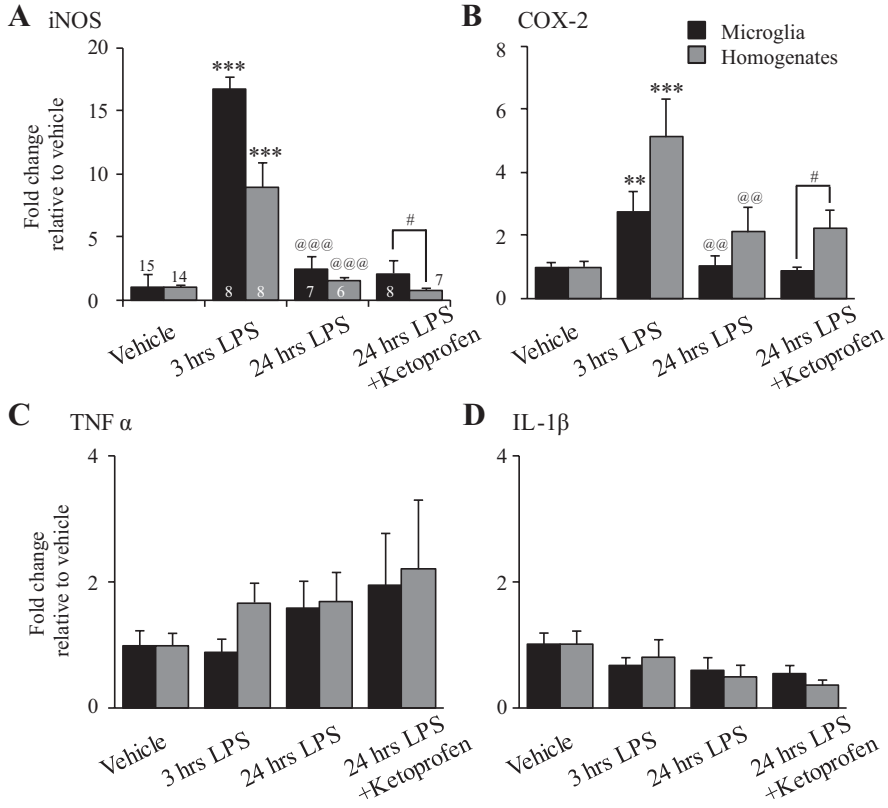

Fig. 4. Systemic inflammation evoked by LPS (100 μg/kg ip) caused transient and differential changes in inflammatory gene expression in isolated microglia (black bars) and homogenates (gray bars) from the cervical spinal cord. *A:* treatment with LPS (3 h) increased mRNA for iNOS compared with vehicle (microglia $n = 15$, homogenates $n = 14$) in both microglia ($n = 8$) and homogenate ($n = 8$) samples. Expression of iNOS was reduced 24 h post-LPS (microglia $n = 7$, homogenates $n = 6$) compared with 3 h post-LPS in both sample types but was not changed relative to vehicle. After ketoprofen (12.5 mg/kg ip, 3 h), microglia had greater iNOS gene expression ($n = 8$) compared with homogenates ($n = 7$). *B:* treatment with LPS (3 h) increased COX-2 mRNA in both microglia ($n = 7$) and homogenate ($n = 8$) compared with vehicle (microglia $n = 15$, homogenates $n = 15$), but was reduced 24 h post-LPS. LPS (24 h) alone (microglia $n = 7$, homogenates $n = 6$) or with ketoprofen (microglia $n = 8$, homogenates $n = 7$) did not alter COX-2 mRNA in either microglia or homogenates compared with vehicle. After ketoprofen treatment, microglia had less COX-2 mRNA compared with homogenates. LPS treatment (3 or 24 h) had no effect on gene expression for TNFα (*C*) or IL-1β (*D*). **$P < 0.01$, ***$P < 0.001$, significant difference from vehicle; @@$P < 0.01$, @@@$P < 0.001$, significant difference from 3 h LPS; #$P < 0.05$, significant difference between microglia and homogenate samples.

Microglia had greater iNOS gene expression vs. homogenates ($P = 0.037$), whereas microglia had less COX-2 mRNA vs. homogenates ($P = 0.011$). Thus microglia may not be the sole contributors to inflammatory molecules in some conditions.

DISCUSSION

Here, we demonstrate that systemic administration of a low dose of LPS impairs AIH-induced pLTF as early as 3 h post LPS injection, an effect that lasts for at least 24 h. However, pLTF impairment is accompanied by only transient (3 h) increases in inflammatory gene expression in the cervical spinal cord. Despite a lack of detectable inflammatory gene expression 24 h post-LPS, systemic administration of the nonsteroidal anti-inflammatory drug ketoprofen (at 21 h) reverses pLTF impairment. We do not yet know the basis for persistent impairment of pLTF without increases in measured inflammatory molecules. Several possibilities are discussed below.

LPS as a model of inflammation. At low doses, LPS activates TLR4/2 receptors, triggering levels of inflammation frequently experienced by humans (62). Since many clinical disorders are associated with low-grade systemic inflammation, low LPS doses may better represent low-grade infections or inflammation and their impact on respiratory plasticity.

In the literature, LPS has been used at a variety of dosages and time points to stimulate systemic inflammation. LPS doses vary from nanograms to milligrams per kilogram (26, 28). LPS effects include behaviors indicative of illness (10, 14, 22), microglial activation, impaired memory, and motor function (32, 57, 61, 64, 66), and changes in neurotrophic factor expression (55). The concentration used here is in the low range reported for rats (16) and elicits reliable systemic and CNS inflammation in mice (62, 72). Higher LPS doses (500 μg/kg or more) typically cause severe systemic inflammation, septic shock, and fever (34, 59, 71), none of which were observed in this study.

Although LPS is often administered systemically to induce CNS inflammation, it does not cross the blood-brain barrier (49, 58). CNS inflammation arises from indirect effects, such as circulating or vascular endothelial cytokines (or other inflammatory molecules) that do cross the blood-brain barrier (49, 58) or neural transmission to the CNS via the vagus nerves (8, 21, 54, 69). Potential molecules crossing the blood-brain barrier to trigger CNS inflammatory activities include the cytokines we assessed in the spleen (e.g., IL-1β and TNF-α) or prostaglandins produced by perivascular macrophages and/or endothelial cells that line the blood-brain barrier (9, 23, 36, 39, 53, 55). However, the precise mechanism(s) of CNS inflammation following LPS injection was not a focus of this study.

We confirmed both systemic and CNS inflammation by examining mRNA levels of selected proinflammatory molecules (iNOS, COX-2, TNFα, and IL-1β). In general, mRNA changes were similar in the spleen and cervical spinal cord. Spleen inflammatory gene expression was greatest 3 h post-LPS (up to 150-fold increase) but had largely returned to baseline expression levels by 24 h post-LPS. We assessed CNS inflammation in cervical spinal segments associated with the phrenic motor nucleus, since cellular mechanisms of pLTF are localized in this region (3, 5, 27, 38). Cervical spinal inflammation was evident in both isolated microglia and homogenates.

Although microglia comprise only a small component of the CNS by volume, they are the predominant immune cells in the CNS (25). To assess microglial LPS responses in the cervical spinal cord, we compared mRNA in spinal homogenates and isolated microglia. Overall, similar trends to the spleen were observed, where iNOS and COX-2 mRNA increased 3- to 15-fold in microglia and homogenates 3 h post-LPS. Although mRNA levels returned nearly to baseline by 24 h post-LPS, we do not have information concerning protein levels for any of the molecules assessed. Collectively, our data suggest that microglia are major contributors to overall changes in inflammatory gene expression in the cervical spinal cord after LPS; however, we cannot rule out important contributions from other cells types, such as astrocytes or neurons.

The time course for increased inflammatory gene expression was shorter than pLTF impairment. Potential explanations include long-lasting changes in the expression of unmeasured molecules that undermine pLTF. Candidate molecules include unmeasured cytokines, interleukins, interferons, chemokines, or other enzymes that initiate distinct signaling cascades. Another possibility is that protein levels of a key molecule outlast mRNA changes (e.g., iNOS or COX-2). A third possibility is that small, persistent elevations in measured molecules were critical in the mechanism undermining pLTF, although not statistically detectable. For example, a critical molecule may need to change very little to undermine pLTF or, more likely, multiple small but undetectable increases (1–2 fold) may act in a cumulative manner, activating a common downstream target molecule that more directly impairs pLTF. All of these possibilities are consistent with ketoprofen restoration of pLTF.

Unlike our previous study using a high LPS dose (67), we observed no change in short-term hypoxic ventilatory response after the low LPS dose used here (either 3 or 24 h post-LPS). Since the magnitude of pLTF correlates with the magnitude of the hypoxic phrenic response in rats (4, 20), there was some concern that the depressed hypoxic phrenic response indirectly impaired pLTF in Vinit et al. (67). Since the hypoxic phrenic response was not affected here, yet pLTF was nevertheless abolished, potential indirect effects are ruled out. Collectively, the data presented here are consistent with the conclusion that a low-grade systemic inflammation suppresses pLTF in rats.

Ketoprofen is a general anti-inflammatory drug used here to confirm that LPS undermines pLTF by inducing inflammation. *S*-enantiomers of ketoprofen inhibit COX-1 and COX-2 at low doses and NFκB at higher doses (13, 73). Ketoprofen inhibits both cyclooxygenase and lipoxygenase pathways for arachidonic acid metabolism, thereby inhibiting synthesis of prostaglandins and leukotrienes (11). Ketoprofen has been given at doses ranging from 100 μg/kg to 50 mg/kg via multiple routes of administration (11, 12, 44, 47). The ketoprofen dose used here was moderately high, making it unclear if its effects were on COX-2 or NFκB. Ketoprofen did not reduce expression of any inflammatory molecules examined; however, we cannot rule out effects of ketoprofen on proteins or other inflammatory molecules not examined. Regardless, our results suggest that a long-lasting, ketoprofen-sensitive molecule significantly impacts AIH-induced pLTF.

The rapid effects of ketoprofen (3 h) in restoring pLTF may implicate COX-2 and prostaglandin synthesis in the impairment of pLTF. These rapid effects are more easily explained by inhibition of COX-2 enzymatic activity (vs. inhibition of NF-κB regulated gene expression). Other studies demonstrate that CNS effects of low-grade systemic inflammation can be dependent on prostaglandins and COX activity (62). Additional studies are necessary to test this idea.

In conclusion, we are only beginning to understand the profound impact of inflammation on respiratory plasticity. The present study highlights the impact of even low-grade systemic inflammation on an important model of respiratory plasticity, AIH-induced pLTF. Further, we provide evidence that microglia, and perhaps other CNS cells, may generate the (as yet unknown) inflammatory molecules that undermine pLTF.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.G.H., S.M.S., J.J.W., and G.S.M. conception and design of research; A.G.H., S.M.S., and S.V. performed experiments; A.G.H., S.M.S., and S.V. analyzed data; A.G.H., S.M.S., J.J.W., and G.S.M. interpreted results of experiments; A.G.H. and S.M.S. prepared figures; A.G.H. drafted manuscript; A.G.H., S.M.S., S.V., J.J.W., and G.S.M. edited and revised manuscript; A.G.H., S.M.S., J.J.W., and G.S.M. approved final version of manuscript.

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