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Blocking Toll-like receptor 2 and 4 signaling during a stressor prevents stress-induced priming of neuroinflammatory responses to a subsequent immune challenge

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Abstract

Acute and chronic stressors sensitize or prime the neuroinflammatory response to a subsequent peripheral or central immunologic challenge. However, the neuroimmune process(es) by which stressors prime or sensitize subsequent neuroinflammatory responses remains unclear. Prior evidence suggested that Toll-like receptors (TLRs) might be involved in the mediation of primed neuroinflammatory responses, but the role of TLRs during a stressor has never been directly tested. Here, a novel TLR2 and TLR4 antagonist, OxPAPC, was used to probe the contribution of TLRs in the stress sensitization phenomenon. OxPAPC has not previously been administered to the brain, and so its action in blocking TLR2 and TLR4 action in brain was first verified. Administration of OxPAPC into the CNS prior to stress prevented the stress-induced potentiation of hippocampal pro-inflammatory response to a subsequent peripheral LPS challenge occurring 24 hours later. In addition, *in vivo* administration of OxPAPC prior to stress prevented the sensitized pro-inflammatory response from isolated microglia following administration of LPS *ex vivo*, further implicating microglia as a key neuroimmune substrate that mediates stress-induced sensitized neuroinflammation.

Keywords

Stress; Microglia; Toll-like receptor; Cytokine; Neuroinflammation; Priming

1. Introduction

Acute and chronic stress sensitize the neuroinflammatory response to subsequent peripheral and central inflammatory challenges, creating an exaggerated neuroinflammatory response (de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Frank et al., 2007; Frank et al., 2010; Johnson et al., 2002; Johnson et al., 2003; Johnson et al., 2004; Munhoz et al., 2006; Wohleb et al., 2011). For example, exposure to a single session of intermittent tailshocks (Johnson et al., 2002) or to chronic unpredictable stress (Munhoz et al., 2006), potentiates the hippocampal and frontal cortical proinflammatory mediators (i.e. interleukin-1 (IL-1)),

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inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α), and nuclear factor kappa b (NF- κ B) activity) induced by a subsequent systemic inflammatory challenge occurring 24 h after the stressor regimen. These inflammatory mediators in the brain are produced predominantly by microglia (Gehrmann et al., 1995), and other studies have shown that both acute and chronic stress activate microglia, as assessed by up-regulated major histocompatibility complex-II (MCHII) (de Pablos et al., 2006; Frank et al., 2007), F4/80 antigen (Nair and Bonneau, 2006; Nair et al., 2007), and microglia proliferation (Nair and Bonneau, 2006). Furthermore, microglia isolated from rats that had received a single session of tail shock 24 h earlier, exhibited up regulated MCHII. Interestingly, these microglia from stressed subjects did not produce increased amounts of pro-inflammatory cytokines (PICs) beyond basal levels. However, if the microglia from stressed rats were stimulated with LPS *ex vivo*, exaggerated amounts of PICs were detected (Frank *et al.*, 2007). This pattern suggests that stress ‘primes’ microglia, as defined by Ransohoff & Perry (Ransohoff and Perry, 2009). That is, the microglia shift to a state in which they are not frankly inflammatory, but produce an exaggerated inflammatory response if stimulated. Taken together, these findings suggest that exposure to a stressor shifts the neuroimmune microenvironment towards a pro-inflammatory state, thereby predisposing certain regions of the CNS to a heightened pro-inflammatory response if the organism is exposed to a subsequent inflammatory challenge.

Secretion of glucocorticoids (GCs) from the adrenals (cortisol in humans and corticosterone (CORT) in rodents) is often taken as a hallmark of the stress response. Since increased levels of GCs are almost universally considered to be anti-inflammatory (Boumpas et al., 1993), the results described above might appear contradictory. However, there is strong evidence demonstrating that GCs can sensitize pro-inflammatory responses, particularly within the CNS (Frank et al., 2010; Frank et al., 2012; Munhoz et al., 2010; Sorrells and Sapolsky, 2007). Replacing the experience of a stressor with a physiologically relevant dose of GCs that mimics the elevated levels of GCs observed during a stressor, produces both exaggerated neuroinflammatory (hippocampus) responses to a systemic LPS challenge 24 hours later (Frank et al., 2010) and ‘primed’ microglia that produce an exaggerated inflammatory response to LPS *ex vivo* (Frank et al., 2012). Further, the glucocorticoid receptor (GR) seems to be critical for GC-induced sensitization. Several studies have shown that stress-induced microglial activation and potentiation of neuroinflammatory processes is blocked by a GC receptor antagonist (de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Munhoz et al., 2006; Nair and Bonneau, 2006). We have demonstrated that blocking GR activity during a stressor with RU486 prevents stress-induced sensitization to a subsequent immune challenge *in vivo*, and the priming of microglia observed *ex vivo* (Frank et al., 2012).

Although the effects of stress-induced sensitization appear to be mediated, at least in part, by increased GC levels, the mechanism(s) whereby stress and GCs sensitize neuroinflammatory responses is largely unknown. Interestingly, GCs upregulate the expression of the pattern recognition receptors (PRR) toll-like receptors (TLR) 2 and TLR4. These PRRs are involved in the recognition of both pathogen associated molecular patterns (PAMPS) and danger associated molecular patterns (DAMPS), and initiate signaling cascades that lead to the synthesis and release of inflammatory mediators (Kawai and Akira, 2007; Salminen et al., 2008). *In vitro* studies have demonstrated that GCs can up-regulate TLR2 expression in epithelial cells via MAPK phosphatase-1 (MKP-1), which in turn inhibits p38 MAPK activity, a negative regulator for TLR2. This increased expression of TLR2 leads to enhanced cytokine expression, including TNF- α , IL-1 β , and IL-8, upon challenge with an inflammatory stimulus (Imasato et al., 2002). Similarly, Rozkova et al., found increased TLR 2 and TLR 4 expression on dendritic cells (DC) following GC treatment (Rozkova et al., 2006). In addition, TNF- α and GCs cooperate to stimulate the promoter for TLR2 and

potentially TLR4, increasing receptor expression (Hermoso et al., 2004). Finally, *in vivo* findings demonstrate that TLR2 mRNA is upregulated 24 h after subcutaneous (SC) injection of GCs (Frank et al., 2010) and TLR4 protein is increased following repeated social stress (Wohleb et al., 2011).

These data suggest that elevated levels of GCs, produced by stress exposure, may sensitize the neuroimmune microenvironment by upregulating expression of TLR2 and TLR4 on CNS innate immune cells. The purpose of the present study was to investigate the involvement of TLR2 and TLR4 during a stressor and assess whether these receptors do mediate the stress-induced sensitized inflammatory response. A novel TLR2 and TLR4 antagonist, Oxidized 1-palmitoyl-2-arachidonyl-sn- glycerol-3-phosphorylcholine (OxPAPC), was used to block TLR2 and TLR4 activity during a stressor. Here we demonstrate that administration of OxPAPC into the CNS prior to stress prevents the exaggerated central (hippocampus) inflammatory response to a subsequent immune challenge. *In vivo* administration of central OxPAPC prior to stress also prevented potentiated inflammatory responses of microglia to LPS *ex vivo*.

2. Methods

2.1 Animals

Male Sprague–Dawley rats (60–90 day-old; Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA) were pair-housed with food and water available *ad libitum*. The colony was maintained at 25 °C on a 12-h light/dark cycle (lights on at 07:00 h). All animals were allowed 1 week of acclimatization to the colony rooms before experimentation. All experimental procedures were conducted in accordance with the University of Colorado Institutional Animal Care and Use Committee.

2.2 Reagents

Lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4) is a TLR4 agonist obtained from Sigma (St. Louis, MO). Lipoteichoic acid (LTA; *Staphylococcus aureus*) is a TLR2 agonist obtained from Invivogen (San Diego, CA). Pam3CSK4 is a TLR1/2 agonist obtained from Invivogen (San Diego, CA). OxPAPC (Invivogen; San Diego, CA) is an oxidized phospholipid that inhibits TLR2 and TLR4 signaling by competitively interfering with extracellular accessory proteins such as CD14, LPS-binding protein (LBP), and MD2 (Erridge et al., 2008). OxPAPC was suspended in 500 μ l chloroform for a lipid concentration of 1 mg/ml and carefully vortexed. The homogeneous solution was aliquoted and evaporated under a stream of nitrogen gas. On the day of experiment, saline was added to create the desired concentration. At higher concentrations, OxPAPC can induce inflammation (Oskolkova et al., 2010). Therefore, an Invivogen recommended concentration of 30 μ g/ml was not exceeded.

2.3 Drug administration

LPS was administered i.p. (10 μ g/kg) or intra-cisterna magna (ICM) (30 ng suspended in 4 μ l sterile saline), depending on experimental design. We selected 10 μ g/kg i.p. LPS because we have previously shown that this dose results in a sub-threshold hippocampal pro-inflammatory response (Johnson et al., 2002). 30ng/4 μ l was selected for ICM administration because pilot studies found that this dose of LPS produces robust pro-inflammatory gene expression as measured by real time RT-PCR in the hippocampus (data not shown).

LTA was administered ICM (40 ng suspended in 4 μ l sterile saline). Similarly, this dose was selected because pilot studies indicated that this dose of LTA produces robust pro-

inflammatory gene expression as measured by real time RT-PCR in the hippocampus (data not shown).

OxPAPC was administered ICM (150ng suspended in 5 μ l sterile saline). *In vivo* and *ex vivo* preliminary work demonstrated that this dose sufficiently inhibited TLR2 and TLR4 activation as measured by proinflammatory gene expression via real time RTPCR (data shown below).

2.4 ICM administration

ICM administration was chosen to deliver drugs centrally because it avoids surgery and canulae implantation, and the long lasting neuroinflammation which results (Holguin et al., 2007). Rats were briefly anesthetized (< 2 min) with halothane. The dorsal aspect of the skull was shaved and swabbed with 70 % ETOH. A 27-gauge needle attached via PE50 tubing to a 25 μ l Hamilton syringe was inserted into the cisterna magna. To verify entry into the cisterna magna, ~ 2 μ l of CSF was drawn. In all cases, CSF was clear of red blood cells indicating entry into the cisterna magna.

2.5 Inescapable tailshock (IS)

Details of the present stressor protocol have been published previously, and the protocol reliably potentiates pro-inflammatory cytokine responses in the hippocampus after peripheral immune challenge (Johnson et al., 2002), as well as in isolated hippocampal microglia to LPS *ex vivo* (Frank et al., 2007). Briefly, animals were placed in Plexiglas tubes (23.4 cm in length $\times$ 7 cm in diameter) and exposed to 100 1.6 mA, 5 s tailshocks with a variable intertrial interval (ITI) ranging from 30–90 s (average ITI = 60 s). All IS treatments occurred between 09:00 and 11:00 h. IS animals were returned to their home cages immediately after termination of shock. HCC animals remained undisturbed in their home cages.

2.6 Tissue collection

Animals were given a lethal dose of sodium pentobarbital. Animals were fully anesthetized and transcardially perfused with ice-cold saline (0.9%) for 3 min to remove peripheral immune cells from the CNS vasculature. Brains were rapidly extracted and placed on ice, and hippocampus dissected. For *in vivo* experiments, hippocampus and liver were flash frozen in liquid nitrogen and stored at -80°C . For *ex vivo* experiments, hippocampal microglia were immediately isolated. Analysis was restricted to the hippocampus because we have shown that it is sensitized to IS and produces robust IS-induced priming effects *in vivo* (Johnson et al., 2002) and *ex vivo* (Frank et al., 2007). Hippocampus also yields a sufficient number of microglia to conduct *ex vivo* experiments. Liver was used as an indicator of peripheral pro-inflammatory responses to inflammatory agents with or without OxPAPC.

2.7 Real time RT-PCR measurement of gene expression

Gene expression was measured using real time RT-PCR. Total RNA was isolated from whole hippocampus utilizing a standard method of phenol:chloroform extraction (Chomczynski and Sacchi, 1987). For detailed descriptions of RNA isolation, cDNA synthesis, and PCR amplification protocols refer to prior publication (Frank et al., 2006). cDNA sequences were obtained from Genbank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed to amplify several cytokines and inflammatory activation markers. Primer sequences were designed using the Qiagen Oligo Analysis & Plotting Tool (oligos.qiagen.com/oligos/toolkit.php?) and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI

(Altschul et al., 1997). Primers were obtained from Invitrogen. Primer specificity was verified by melt curve analysis. Primer sequences are as follows: NFkBIAei, F-CACCAACTACAACGGCCACA, R-GCTCCTGAGCGTTGACATCA, TNF α , F-CAAGGAGGAGAAGTTCCCA, R-TTGGTGGTTTGCTACGACG; IL-1 β , F-CCTTGTGCAAGTGTCTGAAG, R-GGGCTTGAAGCAATCCTTA; IL-6, F-AGAAAAGAGTTGTGCAATGGCA, R-GGCAAATTCCTGGTTATATCC; GAPDH F-TCTTCCAGGAGCGAGATCCC, R-TTCAGGTGAGCCCCAGCCTT. PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA). Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA). Relative gene expression was determined using the 2^{-C_T} (Livak and Schmittgen, 2001). Mean C_T of triplicate measures (C.V. <10%) was computed for each sample. Sample mean C_T of GAPDH (internal control) was subtracted from the sample mean C_T of the respective gene of interest (C_T). The sample with the highest absolute C_T was selected as a calibrator and subtracted from the C_T of each experimental sample (C_T). 2^{-C_T} yields fold change in gene expression of the gene of interest normalized to the internal control gene expression and relative to the calibrator sample.

2.8 Experimental Designs

2.8.1 Effect of OxPAPC on TLR2 & TLR4 signaling *in vitro*—This experiment was a preliminary experiment designed to verify that OxPAPC does function as a TLR2&4 antagonist. We have previously described and used (Hutchinson et al., 2010) a human embryonic kidney-293 (HEK293) cell line stably transfected to express human TLR4 to assess TLR4 activity. This HEK293 cell line expresses high levels of TLR4, the required TLR4 co-signaling molecules (MD-2 and CD14), and an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by several transcription factors such as NF- κ B and AP-1 (Invivogen, San Diego, CA, USA; 293-htlr4a-md2cd14). A parallel HEK-TLR2 (Invivogen, San Diego, CA, USA) cell line was also employed here to examine TLR2 activity. The cells were plated for 48 h in 96 well plates (Microtest 96 well flat bottom plate, Becton Dickinson, Franklin Lakes, NJ, USA; 5×10^3 cells/well) in normal supplement selection media (DMEM with 10% fetal bovine serum (FBS). After 48 hours, supernatant was removed and 160ul of fresh media was added. 20 ul of OxPAPC in different concentrations (5 ug, 10 ug, 20 ug) were added to cells stimulated with 20 ul of LPS (10ng). A TLR 4 ligand, or PAM3CSK4 (100ng), a TLR2 ligand, and incubated for 24 h. Supernatants (15 μ L) were then collected from each well for immediate assay.

TLR2 and TLR4 activity was assessed by measuring the expression of secreted alkaline phosphatase (SEAP) protein. SEAP in the supernatants was assayed using the Phospha-Light System (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. This is a chemiluminescence assay that incorporates Tropic CSPD chemiluminescent substrate. The 15- μ L test samples were diluted in 45 μ L of 1 $\times$ dilution buffer, transferred to 96-well plates (Thermo, Waltham, MA, USA), heated at 65°C in a water bath (Model 210; Fisher Scientific, Pittsburgh, PA, USA) for 30 min, and then cooled on ice to room temperature. Assay buffer (50 μ L/well) was added and, 5 min later, reaction buffer (50 μ L/well) was added and allowed to incubate for 20 min at room temperature. The light output was then measured in a microplate luminometer (#IL213.1191; Dynex Technologies, Chantilly, VA, USA).

2.8.2 Effect of ICM OxPAPC co-administered with ICM LPS or LTA on hippocampal pro-inflammatory cytokine gene expression *in vivo*—Prior studies of OxPAPC have not administered it centrally. To verify that OxPAPC inhibits TLR2 and TLR4 activation in the brain, OxPAPC (150ng/5 μ L, ICM) or vehicle was co-administered

with the TLR2 agonist LTA (40ng/4µl, ICM), the TLR4 agonist LPS (30ng/4µl, ICM) or vehicle, with a 1 µl air bubble separating the two reagents. 2 h after injection of either LPS or vehicle, gene expression of IL-1 and Hippocampus was collected for pro-inflammatory gene mRNA analysis 2 h after injection. The experiment was conducted as two separate cohorts.

2.8.3 Effect of central TLR2 and TLR4 antagonism on peripheral LPS-induced pro-inflammatory cytokine gene expression *in vivo*—Systemically injected LPS does not cross the blood-brain barrier (BBB) (Banks and Robinson, 2010), yet produces robust increases in pro-inflammatory cytokines in the brain and microglia activation markers (Frank et al., 2010). The activating signal that induces this response within the brain remains unknown and may not be dependent on brain TLR4 or TLR2 ligation. To test the involvement of brain TLR2 and TLR4 on CNS pro-inflammatory responses to systemic LPS, OxPAPC (150ng/4µl, ICM) was administered immediately followed by LPS (10µg/kg, i.p.). Hippocampus was collected for inflammatory marker analysis 1 h, 2 h, or 4 h after injection. Since peak inflammatory gene expression occurred 2 h post treatment, liver was also collected at that time point to measure peripheral pro-inflammatory gene expression. To verify that the effects of OxPAPC were mediated within the CNS, OxPAPC (150ng) and LPS (10µg/kg) were injected i.p. Hippocampus and liver were collected 2 h post injection for proinflammatory gene mRNA analysis. The experiment was conducted as two separate cohorts.

2.8.4 Effect of central TLR2 and TLR4 antagonism on stress-induced sensitization of hippocampal pro-inflammatory gene expression to peripheral LPS *in vivo*—To assess whether TLR2 and TLR4 mediate stress-induced sensitized pro-inflammatory cytokine responses, animals were injected with OxPAPC (150ng/4µl, ICM) or vehicle prior to onset of inescapable tailshock (IS) or home cage control (HCC). 24 h post-IS, IS and HCC animals were injected with LPS (10µg/kg, i.p.) or vehicle. Thus, the design was a 2 X 2 X 2 factorial. Two hours post-LPS or vehicle, hippocampal pro-inflammatory cytokines were measured. 2 h post injection was chosen because this was the time at which peak pro-inflammatory cytokine expression was detected in experiment 2.8.3. The experiment was conducted as three separate cohorts.

2.8.5 Effect of central TLR2 and TLR4 antagonism on stress-induced sensitization of hippocampal microglia IL-1β gene expression to LPS *ex vivo*—OxPAPC (150ng/4µl, ICM) or vehicle injections and the IS protocol were identical to those in experiment 2.8.4. Hippocampal microglia from each animal were isolated separately 24 h after stressor termination or HCC using procedures, previously described, that result in highly pure microglia. Hippocampal microglia from each animal were isolated 24 h after stressor termination using procedures, previously described, that result in highly pure microglia (Iba-1+/MHCII+/CD163-/GFAP-) (Frank et al., 2006) with a yield of ~40,000–50,000 cells per hippocampus. Microglia were suspended in DMEM + 10% FBS and microglia concentration for each animal was estimated to be at a density of 10 X 10³ cells/100ul, as determined by trypan blue exclusion. 100µl was added to individual wells of 96-well v-bottom plate. LPS was utilized to challenge microglia *ex vivo* as we have previously determined the optimal *in vitro* conditions under which LPS stimulates a microglia pro-inflammatory cytokine response (Frank et al., 2006). Cells were plated with LPS (0.1, 1.0, 10, 100ng/ml) or media alone for 4 h at 37 °C, 5% CO₂. The 100ng/ml LPS group was excluded from analysis due to cells becoming unviable for unknown reasons in this experiment. The plate was centrifuged at 1000g for 10 min at 4 °C to pellet cells and cells washed 1× in ice cold PBS and centrifuged at 1000g for 10 min at 4 °C. Cell lysis/homogenization and cDNA synthesis was performed according to the manufacturer's

protocol using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen, Carlsbad, CA). The experiment was conducted as three separate cohorts.

2.9 Statistical analysis

All data are presented as mean + SEM. Statistical analyses consisted of ANOVA followed by *t* tests with a Newman-Keuls correction. Threshold for statistical significance was set at = .05. Outliers that were two standard deviations from the mean were removed from analysis. Group numbers are reported in each figure.

3. Results

3.1 Effects of OxPAPC on TLR2 & TLR4 signaling *in vitro*

To verify that OxPAPC inhibits TLR2 & TLR4 activation, NF- κ B-dependent SEAP expression was measured in HEK cells expressing only TLR2 or expressing only TLR4. The data are shown in Supplemental Fig 1. Both Pam₃CSK₄ and LPS significantly increased SEAP expression. Even the high dose of OxPAPC on its own did not have an effect on SEAP expression, but all three concentrations of OxPAPC significantly blunted Pam₃CSK₄ or LPS-induced SEAP expression. A one-way ANOVA was conducted for each group. There was a significant effect in the TLR2 HEK cells ($F_{5,12}=56.06$, $P<.0001$) and TLR4 HEK cells ($F_{5,12}=131.2$, $P<.0001$). Post-hoc analyses showed that OxPAPC significantly reduced expression at concentrations of 5 ($p<.001$), 10 ($p<.001$), and 20 ($p<.001$) μ g/ml in both cell lines. These results validate the efficacy of OxPAPC to inhibit TLR2 and TLR4 signaling *in vitro*.

3.2 Effect of ICM OxPAPC co-administered with ICM LPS or LTA on hippocampal pro-inflammatory cytokine gene expression *in vivo*

A preliminary study was conducted here to assess the efficacy of OxPAPC in blocking TLR2 (Fig.1A.) and TLR4 (Fig.1B.) signaling in the CNS because all previous studies using OxPAPC *in vivo* were limited to peripheral effects. Hippocampal IL-1 α and iB mRNA were measured to determine whether OxPAPC blocked the pro-inflammatory response to a TLR2 agonist (LTA) or a TLR4 agonist (LPS). IL-1 α was measured based on prior evidence indicating brain IL-1 α as the key mediator in neuroinflammatory responses to LPS (Laye et al., 2000). iB mRNA was measured as an indicator of NF- κ B activation, a key transcription factor involved in initiating pro-inflammatory cytokine expression (Brown et al., 1993). The data are shown in Fig. 1. Clearly, both ICM LPS and LTA produced large increases in hippocampal IL-1 α and iB gene expression. Importantly, OxPAPC had no effects of its own, but almost completely blocked the effects of LPS and LTA. The interactions between OxPAPC and LTA (IL-1 α ; $F_{1,20}=14.56$, $p<.01$ and iB ; $F_{1,20}=11.07$, $p<.01$) and OxPAPC and LPS (IL-1 α ; $F_{1,16}=4.92$, $p<.05$ and iB ; $F_{1,17}=12.63$, $p<.01$) were statistically significant. In animals that did not receive OxPAPC, both LTA and LPS significantly increased IL-1 α and iB. Co-administration of OxPAPC blocked LTA and LPS-induced expression of IL-1 α to levels similar to veh/veh groups. Co-administration of OxPAPC blocked LTA-induced expression of iB to levels similar to veh/veh groups. However, Co-administration of OxPAPC only blunted LPS-induced expression of iB but was still significantly increased compared to the veh/veh group. Animals that received OxPAPC/veh did not differ from veh/veh. These results validated the efficacy of OxPAPC to inhibit TLR2 and TLR4 signaling within the brain.

3.3 Effect of central TLR2 and TLR4 antagonism on peripheral LPS-induced cytokine production *in vivo*

To test whether blocking TLR2 and TLR4 activity in the brain would reduce the neuroinflammatory response to systemic LPS, OxPAPC was administered ICM prior to peripheral administration of LPS. Hippocampal IL-1 β (Fig.2A) and iB (Fig.2B) mRNA were examined at three time points (1 h, 2 h, and 4 h) post-treatment. Liver IL-1 β and iB was also measured 2 hr post treatment as an indicator of peripheral inflammatory response (Fig. 2C). Peripheral LPS induced robust increases in hippocampal IL-1 β and iB mRNAs that were evident 1 hr after LPS, and were still present 4 hr after LPS. ICM OxPAPC again had no effects on its own, but completely blocked the inflammatory mRNA increases at the 1 hr timepoint after LPS, and reduced the mRNA increases at the later timepoints, suggesting that the impact of the drug was dissipating. Interestingly, intra-ICM OxPAPC reduced the liver increases produced by the peripheral LPS. A 2×2 (OxPAPC/veh $\times$ LPS/veh) ANOVA was conducted for each time point. In the hippocampus, there was a significant main effect of OxPAPC and LPS on IL-1 β gene expression at 1 hr ($F_{1,16}=8.033$, $p<.05$) and 2 hr ($F_{1,17}=4.991$, $p<.05$) post treatment. Similarly, there was also a main effect on iB at 1 hr ($F_{1,16}=23.02$, $p<.001$) and 2 hr ($F_{1,19}=9.513$, $p<.01$) post treatment. At these time points LPS administered without OxPAPC significantly increased IL-1 β and iB expression, compared to veh/veh and OxPAPC/veh groups. Administration of OxPAPC with LPS significantly reduced IL-1 β and iB mRNAs when compared to the veh/LPS group. Additionally, IL-1 β and iB gene expression did not differ between the OxPAPC/LPS and the veh/veh group. 4 hr post treatment, LPS significantly increased IL-1 β ($F_{1,12}=7.759$, $p<.05$) and iB ($F_{1,12}=54.89$, $p<.001$) gene expression, but there was no interaction between OxPAPC and LPS.

In liver, there was an interaction between OxPAPC and LPS on IL-1 β gene expression ($F_{1,15}=5.547$, $p<.05$). LPS significantly increased IL-1 β compared to veh/veh and OxPAPC/veh groups and administration of OxPAPC prior to LPS significantly decreased the IL-1 β increase produced by LPS alone. iB gene expression increased following LPS ($F_{1,16}=25.11$, $p<.001$), but an interaction between OxPAPC and LPS did not quite reach significance ($F_{1,16}=3.503$, $p=.07$). These results suggest that TLR2 and/or TLR4 within the brain contribute to the inflammatory response within the brain (hippocampus) following a systemic injection of LPS. They also indicate that the peripheral (liver) inflammatory response to LPS is reduced by central administration of OxPAPC. One potential confound is that OxPAPC could cross the BBB to the periphery and prevent peripheral recognition of LPS, thus reducing the inflammatory signal to the CNS. In order to address this issue the dose of centrally administered OxPAPC (150ng) was simultaneously administered i.p. with LPS. 2 h post treatment IL-1 β and iB gene expression were measured in liver and hippocampus. In liver, as shown in Fig. 3, LPS significantly increased IL-1 β ($F_{1,19}=652.5$, $p<.0001$) and iB ($F_{1,19}=143.6$, $p<.0001$), but systemic OxPAPC did not attenuate the effect in either gene. Analysis of Hippocampal tissue displayed similar results. LPS significantly increased IL-1 β ($F_{1,20}=11.96$, $p<.01$) and iB ($F_{1,20}=33.65$, $p<.0001$), and systemic OxPAPC did not reduce this increase. These data suggest that the dose of OxPAPC administered centrally did not functionally inhibit peripheral recognition of LPS by moving to the periphery, since simply injecting this small dose peripherally had no effect.

3.4 Effect of central TLR2 and TLR4 antagonism on stress-induced sensitization of hippocampal pro-inflammatory response to peripheral LPS

The results from 3.3 suggest that peripheral LPS initiates a pro-inflammatory response within the CNS via central TLR2 and/or TLR4. We have previously shown that stressors can potentiate later neuroinflammatory responses to peripheral LPS (Johnson et al., 2002). It has been suggested that stressors might produce this outcome because they act at TLR 2 and/or

TLR4, leading to a sensitized pathway (Frank et al., 2010; Wohleb et al., 2011). In order to test this idea, OxPAPC or vehicle was administered ICM prior to a single session of tail shock or HCC. 24 hours later, LPS or vehicle was injected peripherally and inflammatory markers (IL-1, IL-6, TNF, and iB) in the hippocampus were measured 2 h post injection. We have routinely found that IS alone has no effect on gene expression of inflammatory markers (IL-1, IL-6, and TNF) 24 h after the stressor regime (Frank et al., 2007; Frank et al., 2010; Johnson et al., 2002) and results described above indicate that gene expression for these inflammatory markers does not differ between OxPAPC/veh groups and veh/veh groups. Therefore, OxPAPC/IS/Veh and Veh/IS/Veh groups were omitted from this experiment.

The results are shown in Fig. 4. IS potentiated the increases in IL-1, IL-6, and TNF mRNA produced by peripheral LPS occurring 24 later. ICM OxPAPC given immediately before IS prevented this potentiation. A 2×3 (OxPAPC or Veh $\times$ HCC/Veh or HCC/LPS or IS/LPS) ANOVA was conducted for each gene. Newman-Keuls multiple comparison tests were then applied to genes showing a significant interaction ($p < .05$). There was a significant interaction for IL-1 ($F_{2,33}=3.32, p < .05$) and IL-6 ($F_{2,33}=4.37, p < .05$). As is typical, LPS increased IL-1 and IL-6 gene expression above Veh/HCC/Veh and OxPAPC/HCC/Veh groups, while prior exposure to IS potentiated IL-1 and IL-6 following LPS, relative to animals that only received LPS. Interestingly, pretreatment with OxPAPC prior to IS prevented the exaggerated IL-1 and IL-6 mRNA responses to LPS. Animals that received OxPAPC then IS, and 24 h later received LPS, were significantly different from animals that had received Veh/IS/LPS, and did not differ from Veh/HCC/LPS or OxPAPC/HCC/LPS groups. Importantly, the OxPAPC/HCC/LPS group did not differ from the Veh/HCC/LPS group, demonstrating that OxPAPC is not actively inhibiting the inflammatory response within the hippocampus to systemic LPS 24 h after OxPAPC administration. TNF expression displayed a similar pattern to IL-1 and IL-6 expression, although an interaction between OxPAPC treatment and LPS with or without stress did not quite reach significance ($F_{2,32}=2.93, p = .06$). Given that the pattern of expression for TNF is highly correlated with that of IL-1 and IL-6, and regulations of these genes are closely interconnected, post hoc tests were conducted on TNF gene expression as well. Similar to IL-1 and IL-6, LPS increased TNF expression and exposure to IS potentiated the response to LPS. Administration of OxPAPC prior to IS prevented the exaggerated response to LPS, which was similar to that in animals that did not experience IS. Lastly, there was no interaction for iB gene expression ($F_{2,34}=3.285, p = .25$).

3.5 Effect of central TLR2 and TLR4 antagonism on stress-induced sensitization of hippocampal microglia IL-1 β gene expression to LPS *ex vivo*

We have previously demonstrated that microglia are a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory immune responses (Frank et al., 2007). In order to determine whether OxPAPC prevented stress-induced ‘priming’ of microglial cells, OxPAPC was administered prior to stress and hippocampal microglia were isolated 24 hours post stress. IL-1 gene expression was measured as an indicator of an inflammatory response to LPS based on prior reports suggesting IL-1 as the key mediator in the neuroinflammatory response and “sickness behavior” following LPS exposure (Laye et al., 2000; Luheshi et al., 1996). As can be seen in Fig. 5, LPS increased IL-1 gene expression in a concentration dependent manner in all experimental groups. To determine whether OxPAPC blunted stress-induced sensitization of the microglial IL-1 gene response to LPS challenge, area under the LPS concentration curve (AUC) was computed for each subject as an indicator of the overall LPS response, and a two-way ANOVA determined the interaction between OxPAPC treatment and stress. In HCC animals, IS significantly potentiated the microglial IL-1 response, which was completely blocked by prior OxPAPC treatment

($F_{1,18}=5.651$, $p<.05$). Prior treatment with OxPAPC did not affect IL-1 gene response to LPS in HCC animals.

4. Discussion

The data from the present set of experiments implicate TLR2 and/or TLR4 as a mediator of stress-induced priming of neuroinflammatory responses to subsequent inflammatory challenges. Pharmacological (OxPAPC) antagonism of TLR2 and TLR4 during the experience of stress prevented a primed hippocampal inflammatory response (IL-1, IL-6, and TNF) to a subsequent peripheral LPS challenge 24 h later. In addition, *in vivo* administration of OxPAPC prior to IS prevented the sensitized response to LPS administered directly to isolated microglial cells *ex vivo*, further supporting the idea that microglia are a neuroimmune substrate for stress-induced TLR2 and TLR4 activity. These conclusions are consistent with previous findings demonstrating that microglia become activated or primed following exposure to stress or increased GCs (Espinosa-Oliva et al., 2011; Frank et al., 2007; Frank et al., 2012; Nair and Bonneau, 2006; Wohleb et al., 2011).

The oxidized phospholipid (OxPL), OxPAPC, was used to block TLR2 and TLR4 signaling. In the past, OxPLs were primarily known as augmenters of inflammatory events. However, a recent literature shows that OxPLs possess a wide array of anti-inflammatory effects as well, particularly at lower concentrations (Erridge et al., 2008; Oskolkova et al., 2010; Starosta et al., 2012; von Schlieffen et al., 2009). In particular, OxPAPC has been shown to inhibit TLR2 and TLR4 dependent signaling by competing with the extracellular binding proteins CD-14 and MD-2 at a concentration up to 50 µg/ml, but becomes toxic at higher concentrations (100–300 µg/ml) (Erridge et al., 2008). Further, we have conducted *in vitro* work indicating that OxPAPC directly blocks TLR2 and TLR4 dependent NF- κ B signaling (Supplemental Figure 1). *In vitro* studies have also shown that OxPAPC does not inhibit signaling induced by any other TLR agonist, demonstrating specificity to TLR2 and TLR4 (Erridge et al., 2008). To date, *in vivo* characterization of this drug has been limited to studies within the periphery and it has never been functionally characterized within the CNS. The data from the present set of experiments demonstrates that centrally administered OxPAPC, at a concentration of 30 µg/ml, blocks the neuroinflammatory response (IL-1 & iB) to a centrally administered TLR2 agonist (LTA) and a TLR4 agonist (LPS). Although it is clear that OxPAPC inhibits TLR2 and TLR4 signaling, it is evident that other pathways are involved in the anti-inflammatory effects of OxPAPC. For example, previous studies have shown that OxPAPC can initiate adaptive antioxidant defenses in vascular cells, including activation of the Nrf2 pathway that leads to anti-oxidant response element (ARE) binding of glutamate-cysteine ligase modifier and catalytic (GCLM and GCLC, respectively) and heme oxygenase (HO)-1 (Jyrkkanen et al., 2008). Other transcription factors including activation transcription factor (ATF) 3 are also increased by OxPAPC, particularly at high concentrations (>80 µM) (Oskolkova et al., 2008). As mentioned above, OxPAPC does not interfere with other TLR signaling, demonstrating specificity to TLR2 and TLR4 (Erridge et al., 2008). However, effects at non-TLR locations cannot be ruled out, and this should be noted.

As previously discussed, exposure to acute stress primes the neuroinflammatory response to peripheral LPS. LPS is recognized by TLR4, however, systemic LPS does not cross the BBB. Initial inflammatory responses within the brain can derive from cells at the vascular interface of the BBB and circumventricular organs (Quan et al., 1998; Singh and Jiang, 2004; Vitkovic et al., 2000), which can trigger a series of inflammatory events that result in a sustained neuroinflammatory response. The signaling that maintains inflammation within the brain may not be dependent on TLR4 recognition inside of the parenchyma and is currently not fully understood. In the present study, central administration of OxPAPC

attenuated central (hippocampal) and peripheral (liver) pro-inflammatory gene expression to a simultaneous injection of systemic (ip) LPS. To verify that OxPAPC did not diffuse into the periphery and block initial recognition of LPS, the same dose of OxPAPC was administered ip and was not effective in preventing an inflammatory response. This suggests that TLR2 and/or TLR4 located in the brain is critical for the peripheral-to-central signaling that occurs following peripheral LPS administration. Of course, these data do not address the question of what the ligand(s) within the brain for these receptors might be.

Since the half life of OxPAPC is unknown, one potential confounding factor in the blockade of stress-induced priming found here is that OxPAPC might still have been functional 24 h after administration, and so, was merely attenuating the neuroinflammatory response to the systemic LPS injection, not necessarily preventing stress-induced exaggerated neuroinflammatory responses. It should be noted that the neuroinflammatory response (IL-1, IL-6, and TNF) from HCC animals that received OxPAPC and 24 h later were administered LPS did not differ from the HCC animals that were given a saline injection and 24 h later administered LPS, suggesting that OxPAPC is no longer functional at that time. In addition, an *ex vivo* approach was taken to examine the 'state' of hippocampal microglia following *in vivo* treatment with OxPAPC and IS. Hippocampal microglia were isolated 24 hours after OxPAPC and IS treatment. LPS was used to stimulate the cells *ex vivo* to probe the 'state' of the microglia (i.e., are they sensitized to LPS). Prior administration of OxPAPC prevented the sensitized inflammatory response due to stress, while maintaining the 'normal' inflammatory response to LPS treatment. Since OxPAPC is no longer blocking TLR2 and TLR4 signaling 24 h post injection, the only period of time in which OxPAPC could functionally inhibit TLR2 and TLR4 signaling is during, and directly following, tail shock. This suggests that sometime between the experience of tail shock and the LPS challenge, an unidentified ligand binds to, and activates, TLR2 and/or TLR4, which drives the neuroinflammatory microenvironment to a 'primed' or 'sensitized' state, resulting in exaggerated inflammatory responses if further stimulated, in this case, with LPS.

The present results may help to understand how stressors sensitize inflammatory reactions to a later inflammatory challenge. Although this set of experiments does not identify a potential ligand(s), it does demonstrate that the TLR2 and or TLR4 receptor are likely involved. Interestingly, a new perspective comes from findings that TLRs can be activated by endogenous molecules that are synthesized and secreted in response to "danger". These molecules have been called "alarmins" (Bianchi, 2007; Klune et al., 2008). Alarmins have similar characteristics to PAMPS, such as LPS, meaning that they can activate TLRs and initiate neuroinflammatory responses (Bianchi, 2007). Of these alarmins, HMGB1 is known to activate TLR2 and TLR4 and produce the full array of inflammatory responses, including NF- κ B activity and synthesis of inflammatory cytokines (Mazarati et al., 2011; Park et al., 2004; Yang and Tracey, 2009; Yang et al., 2005). Activation of NF- κ B via TLRs induces the formation of a multiprotein signaling complex known as the inflammasome (Leemans et al., 2011). The inflammasome involves members of the nod-like receptor family (NLRs), with NLRP3 being of particular relevance here. Assembly and activation of the NLRP3 inflammasome is key for cleaving pro-caspase-1 to form the mature and active pro-caspase-1, which in turn cleaves pro-IL-1 to form mature IL-1, resulting in extra-cellular release (Martinon et al., 2009). Formation of the NLRP3 inflammasome requires a 'priming' signal, such as TLR activation, leading to NLRP3 transcription. A secondary signal is required to assemble the inflammasome, leading to IL-1 maturation (Kersse et al., 2011). One possibility is that stress or stress-induced GCs, initiates the 'priming signal' that induces NLRP3 transcription through activity at TLR2 and/or TLR4 via endogenous 'alarmins' such as HMGB-1. A subsequent inflammatory challenge, such as LPS, then assembles the inflammasome resulting in an exaggerated inflammatory response. At this point, this idea is purely speculation. However, there is some evidence the GCs might

function in this way. Busillo et al., found that *in vitro*, GCs increase NLRP3 transcription and protein, thereby priming NLRP3 inflammasome formation to a subsequent stimulus such as LPS or ATP, resulting in a potentiated pro-inflammatory cytokine response (Busillo et al., 2011).

In sum, the present results suggest that exposure to an acute stressor ‘primes’ the CNS innate immune system via a signal that activates TLR2 and/or TLR4. This signal(s) may include endogenous danger signals that are known to be released in response to a wide array of stimuli including infection or sterile injury. Further investigation is required to identify potential signals and determine the cellular processes that drive stress-induced ‘priming’ of innate immune function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997; 25:3389–3402. [PubMed: 9254694]
- Banks WA, Robinson SM. Minimal penetration of lipopolysaccharide across the murine blood-brain barrier. *Brain Behav Immun.* 2010; 24:102–109. [PubMed: 19735725]
- Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007; 81:1–5. [PubMed: 17032697]
- Boumpas DT, Chrousos GP, Wilder RL, Cupps TR, Balow JE. Glucocorticoid therapy for immune-mediated diseases: basic and clinical correlates. *Ann Intern Med.* 1993; 119:1198–1208. [PubMed: 8239251]
- Brown K, Park S, Kanno T, Franzoso G, Siebenlist U. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. *Proc Natl Acad Sci U S A.* 1993; 90:2532–2536. [PubMed: 8460169]
- Busillo JM, Azzam KM, Cidlowski JA. Glucocorticoids sensitize the innate immune system through regulation of the NLRP3 inflammasome. *J Biol Chem.* 2011; 286:38703–38713. [PubMed: 21940629]
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry.* 1987; 162:156–159. [PubMed: 2440339]
- de Pablos RM, Villaran RF, Arguelles S, Herrera AJ, Venero JL, Ayala A, Cano J, Machado A. Stress increases vulnerability to inflammation in the rat prefrontal cortex. *J Neurosci.* 2006; 26:5709–5719. [PubMed: 16723527]
- Erridge C, Kennedy S, Spickett CM, Webb DJ. Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition. *J Biol Chem.* 2008; 283:24748–24759. [PubMed: 18559343]
- Espinosa-Oliva AM, de Pablos RM, Villaran RF, Arguelles S, Venero JL, Machado A, Cano J. Stress is critical for LPS-induced activation of microglia and damage in the rat hippocampus. *Neurobiol Aging.* 2011; 32:85–102. [PubMed: 19286276]
- Frank MG, Baratta MV, Sprunger DB, Watkins LR, Maier SF. Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS proinflammatory cytokine responses. *Brain, Behavior, and Immunity.* 2007; 21:47–59.

- Frank MG, Miguel ZD, Watkins LR, Maier SF. Prior exposure to glucocorticoids sensitizes the neuroinflammatory and peripheral inflammatory responses to *E. coli* lipopolysaccharide. *Brain, Behavior, and Immunity*. 2010; 24:19–30.
- Frank MG, Thompson BM, Watkins LR, Maier SF. Glucocorticoids mediate stress-induced priming of microglial pro-inflammatory responses. *Brain, Behavior, and Immunity*. 2012; 26:337–345.
- Frank MG, Wieseler-Frank JL, Watkins LR, Maier SF. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: Immunophenotypic and functional characteristics. *Journal of Neuroscience Methods*. 2006; 151:121–130. [PubMed: 16125247]
- Gehrmann J, Matsumoto Y, Kreutzberg GW. Microglia: intrinsic immune effector cell of the brain. *Brain Res Brain Res Rev*. 1995; 20:269–287. [PubMed: 7550361]
- Hermoso MA, Matsuguchi T, Smoak K, Cidlowski JA. Glucocorticoids and tumor necrosis factor alpha cooperatively regulate toll-like receptor 2 gene expression. *Mol Cell Biol*. 2004; 24:4743–4756. [PubMed: 15143169]
- Holguin A, Frank MG, Biedenkapp JC, Nelson K, Lippert D, Watkins LR, Rudy JW, Maier SF. Characterization of the temporo-spatial effects of chronic bilateral intrahippocampal cannulae on interleukin-1beta. *J Neurosci Methods*. 2007; 161:265–272. [PubMed: 17241670]
- Hutchinson MR, Loram LC, Zhang Y, Shridhar M, Rezvani N, Berkelhammer D, Phipps S, Foster PS, Landgraf K, Falke JJ, Rice KC, Maier SF, Yin H, Watkins LR. Evidence that tricyclic small molecules may possess toll-like receptor and myeloid differentiation protein 2 activity. *Neuroscience*. 2010; 168:551–563. [PubMed: 20381591]
- Imasato A, Desbois-Mouthon C, Han J, Kai H, Cato AC, Akira S, Li JD. Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontypeable *Haemophilus influenzae*-induced expression of toll-like receptor 2. *J Biol Chem*. 2002; 277:47444–47450. [PubMed: 12356755]
- Johnson JD, O'Connor KA, Deak T, Stark M, Watkins LR, Maier SF. Prior Stressor Exposure Sensitizes LPS-Induced Cytokine Production. *Brain, Behavior, and Immunity*. 2002; 16:461–476.
- Johnson JD, O'Connor KA, Hansen MK, Watkins LR, Maier SF. Effects of prior stress on LPS-induced cytokine and sickness responses. *Am J Physiol Regul Integr Comp Physiol*. 2003; 284:R422–R432. [PubMed: 12399247]
- Johnson JD, O'Connor KA, Watkins LR, Maier SF. The role of IL-1[beta] in stress-induced sensitization of proinflammatory cytokine and corticosterone responses. *Neuroscience*. 2004; 127:569–577. [PubMed: 15283957]
- Jyrkkanen HK, Kansanen E, Inkala M, Kivela AM, Hurttala H, Heinonen SE, Goldsteins G, Jauhiainen S, Tiainen S, Makkonen H, Oskolkova O, Afonyushkin T, Koistinaho J, Yamamoto M, Bochkov VN, Yla-Herttuala S, Levonen AL. Nrf2 regulates antioxidant gene expression evoked by oxidized phospholipids in endothelial cells and murine arteries in vivo. *Circ Res*. 2008; 103:e1–e9. [PubMed: 18535259]
- Kawai T, Akira S. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med*. 2007; 13:460–469. [PubMed: 18029230]
- Kersse K, Bertrand MJ, Lamkanfi M, Vandenabeele P. NOD-like receptors and the innate immune system: coping with danger, damage and death. *Cytokine Growth Factor Rev*. 2011; 22:257–276. [PubMed: 21996492]
- Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A. HMGB1: endogenous danger signaling. *Mol Med*. 2008; 14:476–484. [PubMed: 18431461]
- Laye S, Gheusi G, Cremona S, Combe C, Kelley K, Dantzer R, Parnet P. Endogenous brain IL-1 mediates LPS-induced anorexia and hypothalamic cytokine expression. *Am J Physiol Regul Integr Comp Physiol*. 2000; 279:R93–R98. [PubMed: 10896869]
- Leemans JC, Cassel SL, Sutterwala FS. Sensing damage by the NLRP3 inflammasome. *Immunol Rev*. 2011; 243:152–162. [PubMed: 21884174]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Luheshi G, Miller AJ, Brouwer S, Dascombe MJ, Rothwell NJ, Hopkins SJ. Interleukin-1 receptor antagonist inhibits endotoxin fever and systemic interleukin-6 induction in the rat. *Am J Physiol*. 1996; 270:E91–E95. [PubMed: 8772479]

- Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol.* 2009; 27:229–265. [PubMed: 19302040]
- Mazarati A, Maroso M, Iori V, Vezzani A, Carli M. High-mobility group box-1 impairs memory in mice through both toll-like receptor 4 and Receptor for Advanced Glycation End Products. *Experimental Neurology.* 2011; 232:143–148. [PubMed: 21884699]
- Munhoz CD, Lepsch LB, Kawamoto EM, Malta MB, Lima Lde S, Avellar MC, Sapolsky RM, Scavone C. Chronic unpredictable stress exacerbates lipopolysaccharide-induced activation of nuclear factor-kappaB in the frontal cortex and hippocampus via glucocorticoid secretion. *J Neurosci.* 2006; 26:3813–3820. [PubMed: 16597735]
- Munhoz CD, Sorrells SF, Caso JR, Scavone C, Sapolsky RM. Glucocorticoids exacerbate lipopolysaccharide-induced signaling in the frontal cortex and hippocampus in a dose-dependent manner. *J Neurosci.* 2010; 30:13690–13698. [PubMed: 20943909]
- Nair A, Bonneau RH. Stress-induced elevation of glucocorticoids increases microglia proliferation through NMDA receptor activation. *J Neuroimmunol.* 2006; 171:72–85. [PubMed: 16278020]
- Nair A, Hunzeker J, Bonneau RH. Modulation of microglia and CD8+ T cell activation during the development of stress-induced herpes simplex virus type-1 encephalitis. *Brain, Behavior, and Immunity.* 2007; 21:791–806.
- Oskolkova OV, Afonyushkin T, Leitner A, von Schlieffen E, Gargalovic PS, Lusi AJ, Binder BR, Bochkov VN. ATF4-dependent transcription is a key mechanism in VEGF up-regulation by oxidized phospholipids: critical role of oxidized sn-2 residues in activation of unfolded protein response. *Blood.* 2008; 112:330–339. [PubMed: 18451308]
- Oskolkova OV, Afonyushkin T, Preinerstorfer B, Bicker W, von Schlieffen E, Hainzl E, Demyanets S, Schabbauer G, Lindner W, Tselepis AD, Wojta J, Binder BR, Bochkov VN. Oxidized phospholipids are more potent antagonists of lipopolysaccharide than inducers of inflammation. *J Immunol.* 2010; 185:7706–7712. [PubMed: 21068406]
- Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem.* 2004; 279:7370–7377. [PubMed: 14660645]
- Quan N, Whiteside M, Herkenham M. Time course and localization patterns of interleukin-1 messenger rna expression in brain and pituitary after peripheral administration of lipopolysaccharide. *Neuroscience.* 1998; 83:281–293. [PubMed: 9466417]
- Ransohoff RM, Perry VH. Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol.* 2009; 27:119–145. [PubMed: 19302036]
- Rozkova D, Horvath R, Bartunkova J, Spisek R. Glucocorticoids severely impair differentiation and antigen presenting function of dendritic cells despite upregulation of Toll-like receptors. *Clin Immunol.* 2006; 120:260–271. [PubMed: 16765091]
- Salminen A, Huuskonen J, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T. Activation of innate immunity system during aging: NF- κ B signaling is the molecular culprit of inflamm-aging. *Ageing Res Rev.* 2008; 7:83–105. [PubMed: 17964225]
- Singh AK, Jiang Y. How does peripheral lipopolysaccharide induce gene expression in the brain of rats? *Toxicology.* 2004; 201:197–207. [PubMed: 15297033]
- Sorrells SF, Sapolsky RM. An inflammatory review of glucocorticoid actions in the CNS. *Brain, Behavior, and Immunity.* 2007; 21:259–272.
- Starosta V, Wu T, Zimman A, Pham D, Tian X, Oskolkova O, Bochkov V, Berliner JA, Birukova AA, Birukov KG. Differential regulation of endothelial cell permeability by high and low doses of oxidized 1-palmitoyl-2-arachidonoyl-snglycero- 3-phosphocholine. *Am J Respir Cell Mol Biol.* 2012; 46:331–341. [PubMed: 21997484]
- Vitkovic L, Konsman JP, Bockaert J, Dantzer R, Homburger V, Jacque C. Cytokine signals propagate through the brain. *Mol Psychiatr.* 2000; 5:604–615.
- von Schlieffen E, Oskolkova OV, Schabbauer G, Gruber F, Bluml S, Genest M, Kadl A, Marsik C, Knapp S, Chow J, Leitinger N, Binder BR, Bochkov VN. Multi-hit inhibition of circulating and cell-associated components of the toll-like receptor 4 pathway by oxidized phospholipids. *Arterioscler Thromb Vasc Biol.* 2009; 29:356–362. [PubMed: 19112167]

- Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, Nelson RJ, Godbout JP, Sheridan JF. beta-Adrenergic Receptor Antagonism Prevents Anxiety-Like Behavior and Microglial Reactivity Induced by Repeated Social Defeat. *Journal of Neuroscience*. 2011; 31:6277–6288. [PubMed: 21525267]
- Yang H, Tracey KJ. Targeting HMGB1 in inflammation. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*. 2009; 1799:149–156.
- Yang H, Wang H, Czura CJ, Tracey KJ. The cytokine activity of HMGB1. *J Leukoc Biol*. 2005; 78:1–8. [PubMed: 15734795]

Highlight

Pharmacological antagonism of TLR2 and TLR4 signaling during a stressor abrogated the stress-induced sensitized neuroinflammatory response to an immune challenge.

Figure 1.

Effects of OxPAPC (150ng/5 μ l;ICM) on hippocampal pro-inflammatory gene expression following ICM administration of a (A) TLR2 agonist (LTA; 40ng/4 μ l) or a (B) TLR4 agonist (LPS;30ng/4 μ l). Animals received simultaneous ICM administration of OxPAPC and TLR agonist. Hippocampus was collected 2 h post-injection for pro-inflammatory gene expression analysis. Means with different letters are significantly different ($p < .05$). Data are presented as mean $\pm$ SEM. $N=5-6$ animals/group.

Figure 2.

Effects of OxPAPC (150ng/5 μ l;ICM) on pro-inflammatory gene expression following systemic LPS (10 μ g/kg;ip) administration. Hippocampal (A) IL-1 and (B) κ B gene expression were measured 1 h, 2 h, and 4 h post treatment. Since the peak inflammatory response was 2 h post-LPS treatment, this time point was chosen to examine (C) IL-1 and (D) κ B gene expression in the liver. Means with different letters (a,b,c) are significantly different ($p < .05$). Data are presented as mean $\pm$ SEM. $N=4-6$ animals/group.

Hippocampus

Liver

Figure 3. Effects of OxPAPC (150ng;ip) on pro-inflammatory gene expression following LPS (10 μ g/kg;ip) treatment. Tissue was collected 2 h post treatment. Means with different letters are significantly different ($p < .05$). Data are presented as mean $\pm$ SEM. $N=5-6$ animals/group.

Figure 4.

Effects of the TLR2 and TLR4 antagonist OxPAPC on stress-induced sensitized pro-inflammatory response to LPS in hippocampus. Animals were pretreated with OxPAPC (150ng/5 μ l;ICM) or vehicle. Animals were then exposed to inescapable tailshock (IS) or served as home cage controls (HCC). Animals were injected with LPS (10 μ g/kg;i.p.) or vehicle twenty four hours post-IS exposure. Hippocampal IL-1, IL-6, and TNF were measured two hours post-LPS or vehicle injection. Means with different letters are significantly different ($p < .05$). Data are presented as mean $\pm$ SEM. $N=6-8$ animals/group.

Figure 5.

Effects of OxPAPC on stress-induced sensitization of microglial IL-1 response to LPS *ex vivo*. Animals were pretreated with OxPAPC (150ng/5 μ l;ICM) or vehicle. Animals were then exposed to inescapable tailshock (IS) or served as home cage controls (HCC). Twenty four hours post-IS exposure, microglia were isolated from hippocampus and challenged with LPS (0, 0.1, 1, and 10 ng/ml) for 4 h and microglial IL-1 gene expression was measured. LPS increased IL-1 gene expression in a concentration dependent manner in all experimental groups. To determine whether OxPAPC treatment blunted stress-induced sensitization of the microglial IL-1 response to LPS challenge, area under the LPS concentration curve (AUC) was computed for each animal and means compared. Means with different letters are significantly different ($p < .05$). Data are presented as mean $\pm$ SEM. $N=5-6$ animals/group.