Glucocorticoids Exacerbate Lipopolysaccharide-Induced Signaling in the Frontal Cortex and Hippocampus in a Dose-Dependent Manner

Carolina Demarchi Munhoz,^{1,2} Shawn F. Sorrells,¹ Javier R. Caso,¹ Cristoforo Scavone,² and Robert M. Sapolsky¹

¹Department of Biological Sciences, Stanford University, Stanford, California 94305-5020, and ²Department of Pharmacology, Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil 05508-900

Although the anti-inflammatory actions of glucocorticoids (GCs) are well established, evidence has accumulated showing that proinflammatory GC effects can occur in the brain, in a poorly understood manner. Using electrophoretic mobility shift assay, real-time PCR, and immunoblotting, we investigated the ability of varying concentrations of corticosterone (CORT, the GC of rats) to modulate lipopolysaccharide (LPS)-induced activation of NF- κ B (nuclear factor κ B), expression of anti- and proinflammatory factors and of the MAP (mitogen-activated protein) kinase family [ERK (extracellular signal-regulated kinase), p38, and JNK/SAPK (c-Jun N-terminal protein kinase/stress-activated protein kinase)], and AKT. In the frontal cortex, elevated CORT levels were proinflammatory, exacerbating LPS effects on NF- κ B, MAP kinases, and proinflammatory gene expression. Milder proinflammatory GCs effects occurred in the hippocampus. In the absence of LPS, elevated CORT levels increased basal activation of ERK1/2, p38, SAPK/JNK, and AKT in both regions. These findings suggest that GCs do not uniformly suppress neuroinflammation and can even enhance it at multiple levels in the pathway linking LPS exposure to inflammation.

Introduction

Inflammation is a complex, specialized immune response to a pathogen or traumatic event. Whereas inflammation can be beneficial, exaggerated inflammation can be adverse (Medzhitov, 2008).

Glucocorticoids (GCs), secreted by the adrenal glands during stress, are well known to be anti-inflammatory and immunosuppressive, providing the rationale behind their frequent clinical use. Anti-inflammatory GC actions include blunting expression of proinflammatory genes (e.g., *TNF- α* and *IL-1 β*), increasing expression of anti-inflammatory genes (e.g., *I κ B- α* and *IL-1ra*), and, perhaps most importantly, inhibiting activity of NF- κ B (nuclear factor κ B) (De Bosscher et al., 2000; Almawi and Melemedjian, 2002). NF- κ B is a transcription factor constitutively expressed in the cytoplasm and, in the absence of stimulus, bound to I κ B- α , which masks the nuclear localization signal of NF- κ B (Ghosh et al., 1998). Lipopolysaccharide (LPS), IL-1 β , TNF- α (tumor necrosis factor), and reactive oxygen species all induce NF- κ B by activating I κ B kinases. These kinases phosphorylate I κ B- α , leading to its polyubiquitination and degradation at the proteasome

26S (Ghosh and Karin, 2002), allowing NF- κ B to migrate to the nucleus and activate transcription of proinflammatory genes. GCs induce I κ B- α expression, preventing nuclear translocation of NF- κ B (Aljada et al., 1999; Quan et al., 2000), and interact with the NF- κ B p65 subunit, thereby blocking NF- κ B-DNA-binding activity (Unlap and Joep, 1997; De Bosscher et al., 2000; McKay and Cidlowski, 2000).

LPS activates the innate immune system by generating and releasing inflammatory mediators essential for the early innate and subsequent adaptive host defense. LPS binds to Toll-like receptor 4 (TLR4), which recruits the receptor-associated adapter protein MyD88 pathway, culminating in activation of NF- κ B and/or activation of mitogen-activated protein kinase (MAPK) (Konat et al., 2006). In the brain, similar activation can be induced by cellular stress signals (e.g., UV irradiation or osmotic shock) and proinflammatory cytokines (Karin, 1998). In contrast, the PI3K/AKT pathway has emerged as a negative regulator of TLR4 signaling and an important player in establishing endotoxin tolerance in macrophages (Zhang and Daynes, 2007a).

Nonetheless, an emerging literature shows that, counter to expectations, there are circumstances following an inflammatory challenge or insult where GCs or stress enhance rather than blunt inflammation (for review, see Sorrells et al., 2009). Consonant with this, we have shown that chronic unpredictable stress potentiates LPS-induced NF- κ B activation and proinflammatory cytokine expression in frontal cortex and hippocampus, but not in hypothalamus of rats. The proinflammatory effects of stress were mediated by glucocorticoid receptors (GR) since the pretreatment with the GR antagonist RU-486 blunted the stress effects on NF κ B potentiation (Munhoz et al., 2006).

Received Jan. 19, 2009; revised Dec. 20, 2009; accepted Dec. 26, 2009.

This work was supported by research grants to C.S. from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP: 2002/02298-2 and 2004/11041-0). C.D.M. was supported by FAPESP (04/11042-7); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq: 201059/2003-7) and Adler Foundation. C.S. is a research fellow from CNPq. J.R.C. is supported by MICINN (I-D + I 2008-2011) postdoctoral fellowship. National Institutes of Health Grants R01 MH53814 and P01 NS37520 were awarded to R.M.S. We gratefully thank Larissa de Sá Lima for technical assistance and Nathan Manley, Melissa Works, Michelle Cheng, and Lucilia Lepsch for helpful discussions.

The authors report no biomedical financial interests or potential conflicts of interest.

Correspondence should be addressed to Carolina Demarchi Munhoz, Department of Biology, 371 Serra Mall, Gilbert Building, Stanford University, Stanford, CA 94305-5020. E-mail: cdmunhoz@usf.br.

DOI:10.1523/JNEUROSCI.0303-09.2010

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Although it is becoming increasingly evident that proinflammatory CORT effects can occur in the brain, the mechanisms by which these unexpected effects occur are poorly understood. In this study we examined the dose–response relationship between the levels of CORT and their proinflammatory effects on LPS-induced activation of NF-κB, and on the mRNA levels of anti- and proinflammatory factors in the frontal cortex and hippocampus of rats. Moreover, we investigated the status of MAPK family [ERK, p38, and JNK/SAPK (c-Jun N-terminal protein kinase/stress-activated protein kinase)] and AKT activation under LPS treatment and different levels of CORT.

Materials and Methods

Chemicals and kits. LPS (from *Escherichia coli* O111:B4) and CORT were purchased from Sigma Chemical, γ-³²P-ATP and poly dI-dC from GE Healthcare, the gel shift assay system kit for NF-κB from Promega, and the protein assay kit from Bio-Rad.

Glucocorticoid manipulation and LPS injections. All experiments were conducted under the guidelines described in the US Public Health Service Policy on Human Care and Use of Laboratory Animals and with the ethical principles in animal research adopted by the Biomedical College of Animal Experimentation.

Adult male Sprague Dawley rats (280–320 g; from Charles River Laboratories) were kept under a 12 h light/dark cycle (lights on at 7 A.M.) and fed *ad libitum*. Four groups were used, with each group administered a different concentration of CORT. In the control group [intact (INT)], neither adrenalectomy nor GC manipulations were performed; thus animals had basal GC levels (~1–10 μg/dl). In another group, rats were bilaterally adrenalectomized under isoflurane anesthesia and a CORT pellet was implanted subcutaneously. CORT pellets (15, 30, or 100%) were implanted to generate basal CORT levels (15%, 6.0 μg/dl) or levels of CORT typical of a mild stressor (30%, 18–20 μg/dl) or a moderate stressor (100%, 28–32 μg/dl), respectively (Stein-Behrens et al., 1994; Sapolsky et al., 1995). Rats were given 3 d for hormone levels to stabilize before LPS or SAL injection. In the 100% CORT group, levels were raised into the range of major stressors for ~20 h/d by injecting rats daily for 3 d with 10 mg of CORT/d s.c. in sesame oil (Dinkel et al., 2003). All adrenalectomized rats were kept under 0.9% saline as drinking water. Four days after adrenalectomy, INT and 15%, 30% and 100% CORT groups were treated with either saline or LPS dissolved in saline (1 mg/kg, intravenous bolus), and killed by decapitation 2 h later, when maximal NF-κB binding activation is obtained in different brain regions (Glezer et al., 2003). The brain was removed and immersed in cold PBS. The frontal cortex and hippocampus were rapidly dissected, quickly immersed in liquid nitrogen, and stored at –80°C.

Serum CORT concentration. Concentrations of CORT in serum were quantified by using an enzyme-linked immunoassay (Assay Designs). Trunk blood was collected in 15 ml conical tubes and centrifuged at 3000 rpm for 10 min to obtain serum. CORT titers were assessed by using a competitive enzyme immunoassay kit, following the manufacturer's instructions.

Tissue preparation and Western blotting. Nuclear extracts of each brain structure were prepared as described previously (Munhoz et al., 2006). Briefly, brain structures were homogenized in cold PBS and centrifuged for 30 s at 4°C at 14,000 rpm. The supernatants from this first step (S1) were kept on ice. The pellets were resuspended in lysis buffer, kept on ice for 10 min, and centrifuged for 30 s at 4°C at 14,000 rpm. The resulting supernatants (S2) were mixed with S1 and used as cytosolic fraction for Western blot assays. The pellets, containing the nuclear fraction, were resuspended in extraction buffer, kept on ice for 20 min, and centrifuged at 12,000 rpm at 4°C for 20 min. The resulting supernatants containing nuclear proteins were stored at –80°C. Protein concentration was determined using the Bradford method (Bio-Rad) (Bradford, 1976).

Electrophoresis was performed using 10% polyacrylamide in a Bio-Rad mini-Protein III apparatus. In brief, proteins present in the cytosolic fraction were boiled (95°C for 5 min) and size-separated in 10% SDS polyacrylamide gels (90 V). Proteins (30 μg) were blotted onto a PVDF membrane (Bio-Rad) and incubated with specific antibodies: Thr202/Tyr204-phospho-ERK, Thr183/Tyr185-phospho-SAPK/JNK (Cell Sig-

Table 1. Primer sequences used in the quantitative PCR assays

Primer	Sequence
TNF-α	FW: TCTGCTTGGTGGTTTGCTACGAC RV: TGAGTACCTGGACTTGCAGAACG
IL-1β	FW: GGGTTCATGGTGAAGTCAAC RV: AAGACCTTCTACCTGAGGAACAACC
IκB-α	FW: TGTAGATGCCCTTCCAAGGATGG RV: CTCATGGACTGATTATGGACAGGAC
IL1ra	FW: GCCAAGAACACATTCCGAAAGTC RV: AAATGGGCTCCCTCTCATCAGTTC
MKP-1	FW: ACTTCCTGTCTCCATCCCT RV: ACGTGAACTCCACACCTCC
HPRT	FW: CTCATGGACTGATTATGGACAGGAC RV: GCAGGTGACGAAGAAGCTTATAGCC

FW, Forward; RV, reverse.

Figure 1. Serum CORT levels produced by CORT/cholesterol pellets in rats treated with saline (SAL) or LPS. Results are presented as mean ± SEM (n = 6 animals per group). Bonferroni test: *p < 0.05; **p < 0.01; ***p < 0.001.

naling Technology) at a dilution of 1:2000, and Thr180/Tyr182-phospho-p38 (Abcam) at a dilution of 1:1000 for 2 h at room temperature; Ser473-p-AKT (Millipore Bioscience Research Reagents) at a dilution of 1:500, overnight at 4°C. To ensure equal protein loading, we used the Ponceau method to stain the membranes before probing with the antibodies (Salinovich and Montelaro, 1986). Proteins recognized by antibodies were revealed by ECL Plus reagent, following manufacturers' instructions (GE Healthcare, Pfizer). β-actin antibody (Santa Cruz Biotechnology) was used as an internal control for the cytosolic protein fraction and Lamin B1 antibody (Abcam) was used as an internal control for the nuclear protein fraction.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) to NF-κB was performed by using the gel shift assay kit from Promega, as previously described (Rong and Baudry, 1996). ³²P-NF-κB double-stranded consensus oligonucleotide probe (5'-AGTTGAGGGGA-CTTCCCCAGGC-3') 20,000 cpm and nuclear extracts (10 μg) were used. DNA-protein complexes were separated by electrophoresis through a 6% nondenaturing acrylamide:bis-acrylamide (37.5:1) gel in 0.5 × Tris-borate/EDTA (TBE) for 2 h at 150 V. Gels were vacuum-dried, and analyzed by autoradiography. For competition experiments, NF-κB and TFIIID (Transcription factor IID, 5'-GCAGAGCATATAAG-GTGAGGTAGGA-3') unlabeled double stranded consensus oligonucleotide was included in 15-fold molar excess over the amount of ³²P-NF-κB probe to detect specific and nonspecific DNA-protein interactions, respectively. Supershift assays, using antibodies against different NF-κB subunits (p50 and p65, 1:20 dilution; cRel, 1:10 dilution) were also conducted according to manufacturer's protocol (Santa Cruz Biotechnology).

Real-time quantitative PCR. Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) was performed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories) with random and oligo dT primers mixture. To

avoid genomic DNA contamination, total RNA (2 μ g) was treated with deoxyribonuclease I (DNase I) and RNase-free enzyme (Fermentas) before RT reactions. Real-time PCR was performed according to the IQ Sybr Green Supermix protocol (Bio-Rad Laboratories), and amplification was performed in a Rotor-Gene 6000 (Corbett Research). Amplicons that span over two or more exons were selected, whenever possible, to distinguish between amplification of mature mRNA from that of genomic DNA. Relative gene expression was calculated from cycle threshold values (Ct) following a protocol previously described (Dussault and Pouliot, 2006). *HPRT* gene (hypoxanthine-guanine phosphoribosyltransferase) was used as an internal control for each individual sample gene expression. The corresponding sequences for each gene are listed in Table 1.

Statistical analysis. Autoradiographs from EMSA and Western blot assays were analyzed with the Gel Documentation System Doc-Print (Vilber Lourmart) and ImageJ software (National Institutes of Health, Bethesda, MD). All results are expressed as mean $\pm$ SEM. Statistical comparisons were performed in each brain structure individually using two-way ANOVA (CORT levels $\times$ LPS) followed by Bonferroni *post hoc* test (GraphPad Prism5 software package). Significance for all tests was set at $p < 0.05$.

Results

To validate the GC manipulation used in this study, we measured CORT levels in serum of all animals. As expected, CORT/cholesterol pellets produced circulating CORT concentrations that varied as a function of the percentage CORT in each pellet (Fig. 1): INT and 15% CORT rats had basal CORT concentrations ($<5 \mu$ g/dl); 30% pellets produced values mimicking the mild stress range (16–18 μ g/dl); and 100% pellets with the 10 mg/kg CORT daily injections produced values close to 30 μ g/dl, mimicking concentrations found in response to moderate to substantial stressors. LPS caused an increase in serum CORT levels only in INT group (25 μ g/dl), producing CORT levels found in response to mild stress. As expected, adrenalectomy blocked CORT elevation induced by LPS in all animals independently of the CORT pellet implanted.

CORT potentiates LPS-induced NF- κ B binding activity in frontal cortex and hippocampus

As expected, LPS activated NF- κ B binding activity within 2 h in the frontal cortex and hippocampus; in the frontal cortex, NF- κ B activation was augmented in animals receiving 30% CORT pellets, but was decreased in 100% CORT pellet animals (Fig. 2A). In the hippocampus, 15% CORT and 30% CORT pellets enhanced the LPS-induced NF- κ B activation (Fig. 2B). In the absence of LPS, none of the CORT pellets groups altered NF- κ B binding activity in either brain region. An excess of unlabeled NF- κ B oligonucleotide probe completely eliminated the observed NF- κ B bands, demonstrating that these bands correspond to specific binding. In addition, super shift experiments confirmed the

Figure 2. Influence of CORT levels on NF- κ B activation induced by LPS or saline (SAL) in the frontal cortex and hippocampus. Top, Representative EMSA autoradiography of frontal cortex (A) and hippocampus (B). NF- κ B-specific band is indicated by arrow. Representative autoradiography from Western blot assay of the translocation of p65-NF- κ B subunit in frontal cortex (C) and hippocampus (D). Bottom, Densitometric analysis of the NF- κ B-specific band (representing p65/p50 heterodimers) or cytosolic and nuclear ratio presented in the top (mean $\pm$ SEM; $n = 5$ animals per group). Bonferroni test: ** $p < 0.01$; *** $p < 0.001$.

presence of p50 and p65 in the protein complexes bound to the radiolabeled NF- κ B probe (Fig. 3A,B).

To further explore CORT's effect on NF- κ B activation, the nuclear expression of the NF- κ B subunit p65 was examined by Western blot analysis. LPS caused translocation of this subunit to the nucleus in frontal cortex (Fig. 2C) and hippocampus (Fig. 2D), and this effect was potentiated by mid to high CORT levels.

CORT potentiates LPS-induced expression of proinflammatory genes *TNF- α* and *IL-1 β*

We next examined whether CORT also potentiated the effects of LPS upon expression of proinflammatory genes known to be induced by NF- κ B (i.e., *TNF- α* and *IL-1 β*). In the absence of LPS, CORT had no effect on the expression of proinflammatory genes in the frontal cortex and hippocampus (Fig. 4).

As expected, in INT rats, LPS increased expression of these proinflammatory genes in frontal cortex and hippocampus. As with NF- κ B activation as an endpoint, high CORT levels also potentiated the LPS effects on mRNA expression of all four genes in the frontal cortex and hippocampus (Fig. 4). CORT showed an inverse-U pattern (i.e., mid-range CORT levels have the opposite effect of very low and very high) of proinflammatory effects (e.g.,

Figure 3. Competition studies and supershift assays were performed on nuclear extract from frontal cortex (A) and hippocampus (B) of rats implanted with CORT pellets and treated with saline or LPS (1 mg/kg, i.v., 2 h) in the absence or presence of unlabeled specific (NF-κB consensus sequence, 15-fold molar excess) or nonspecific (TFIID consensus sequence, 15-fold molar excess) oligonucleotide, as indicated. Supershift assays were performed with the same nuclear extract (10 μg) incubated in the absence and presence of antibodies against subunits p50 (1:20 dilution), p65 (1:20 dilution), and cRel (1:20 dilution), as indicated. The position of specific NF-κB/DNA binding complex p50/p65 is indicated. NS represents nonspecific binding. The localization of the probe is also indicated. Results are representative of three independent experiments.

Figure 4. Influence of CORT levels on proinflammatory genes (*IL-1β* and *TNF-α*) in the absence (SAL) or presence of LPS in the frontal cortex (A, C) and hippocampus (B, D). Real-time PCRs were performed in duplicate and results are represented as mean ± SEM; n = 6 animals per group. Bonferroni test: *p < 0.05; **p < 0.01; ***p < 0.001 vs INT/LPS.

TNF-α in the frontal cortex) and a progressive increase in *IL-1β* with increasing CORT levels (e.g., in the frontal cortex).

Thus, in the absence of LPS, CORT had no effects in both brain regions; in contrast, LPS-induced expression of *TNF-α* and *IL-1β* was consistently augmented by elevated CORT levels.

CORT fails to increase anti-inflammatory genes *IL-1ra*, *IκB-α*, and *MKP-1* expression

The observed proinflammatory GCs effects in the brain were unexpected, which prompted us to investigate whether CORT levels would have unexpected effects on the expression of anti-inflammatory genes such as *IκB-α*, *IL-1ra*, and *MKP-1*, classically known to be upregulated by GCs.

In the absence of LPS, high CORT levels generally increased levels of these anti-inflammatory genes in both brain regions (Fig. 5). Thirty percent and 100% CORT increased the expression of *IκB-α* and *MKP-1* in the frontal cortex (Fig. 5A,E) and had no effects on *IL-1ra* mRNA expression (Fig. 5C). In the hippocampus, mid to high (30% and 100%) CORT levels increased *IκB-α* and *MKP-1* mRNA expression (Fig. 5B,F) but, as in the frontal cortex, CORT had no effect on the expression of *IL-1ra* (Fig. 5D).

As expected, LPS treatment altered the expression of these anti-inflammatory genes. For *IκB-α* and *IL-1ra*, LPS increased expression in both regions. However, for *MKP-1*, LPS increased expression in the hippocampus but had no effect in the frontal cortex (Fig. 5E,F).

For these anti-inflammatory genes, CORT had varying effects on LPS-induced gene expression. Some CORT effects were classically anti-inflammatory (i.e., *MKP-1* in the hippocampus), yet in some instances CORT failed to produce anti-inflammatory effects (i.e., *IL-1ra* in both regions). In one case, CORT effects were proinflammatory (i.e., *IκB-α* in the cortex), and in another, a mixture of pro and anti-effects that formed an inverse-U curve (i.e., *IκB-α* in the hippocampus).

CORT exacerbates LPS effects on activation of MAPK family (ERK-1 and 2, p38, and SAPK/JNK) and AKT

As was mentioned before, the MAPK family (e.g., ERK 1/2 [MAPK 42/44], SAPK/JNK, p38 kinase) and protein kinase AKT play an important role in mediating the immune response and are modulated by LPS. We next tested whether CORT could modify the LPS effects on their activation, as manifested by these kinases being phos-

phorylated. Thus, we measured the levels of the phosphorylated forms of these kinases.

In the absence of LPS, progressively higher levels of CORT increased the activation of p38 and SAPK/JNK in the frontal cortex and hippocampus, and high CORT levels increased activation of ERK 1 and 2 in the hippocampus with no effect in the frontal cortex. In addition, basal levels of CORT increased the activation of AKT in the hippocampus, while elevated CORT levels increased AKT activation in the frontal cortex (Figs. 6 and 7).

As expected, LPS treatment activated all kinases analyzed in this study in both brain areas when compared with the intact animals that received saline. In the frontal cortex, high CORT levels (100%) blunted the LPS-induced activation of ERK and p38 kinases (Fig. 6*A,B*). In the hippocampus, while mid-range CORT levels potentiated and high CORT levels blocked LPS effects on ERK activation, both mid and high CORT levels potentiated LPS effects on p38 kinase activation (Fig. 7*A,B*).

For the SAPK/JNK activities, while basal and high levels of CORT blunted LPS effects in the frontal cortex (Fig. 6*C*), basal to mid-range levels potentiated the LPS-induced activation of this kinase in the hippocampus and high CORT levels blunted the LPS effects (Fig. 7*C*).

Finally, CORT had no effect on the LPS effect in AKT phosphorylation in the frontal cortex (Fig. 6*D*) and in the hippocampus (Fig. 7*D*).

Thus, in the absence of LPS, elevated CORT levels progressively increased the activation of ERK 1/2, p38, SAPK/JNK, and AKT in the frontal cortex and hippocampus. In contrast, LPS-induced activation of these kinases was modulated in a number of different ways by increased CORT levels, with similar effects in frontal cortex and hippocampus.

Discussion

Elevated CORT potentiates

LPS-induced NF- κ B activation

GC inhibition of NF- κ B activity is central to the hormone's anti-inflammatory actions. GCs can accomplish this by increasing expression of I κ B- α , and by inhibiting NF- κ B binding to DNA, via interactions between activated GR and the p65-NF- κ B subunit (McKay and Cidlowski, 2000). However, contrary to the picture of GCs as uniformly anti-inflammatory, we find that GCs failed to increase expression of some anti-inflammatory genes in the brain in the presence of LPS; to our knowledge, this is the first such report. Even more strikingly, we find that in some situations, GCs actually enhance aspects of inflammation (Table 2). Mid to high CORT levels exacerbated LPS-induced NF- κ B activation and proinflammatory gene expression in the frontal cortex

Figure 5. Influence of CORT levels on anti-inflammatory genes (I κ B- α , IL-1ra, and MKP-1) in the absence (SAL) or presence of LPS in the frontal cortex (*A, C, E*) and hippocampus (*B, D, F*). Real-time PCRs were performed in duplicate and results are represented as mean $\pm$ SEM; $n = 6$ animals per group. Bonferroni test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. For MKP-1: ### $p < 0.001$ vs INT/SAL; ** $p < 0.05$ vs INT/LPS.

and hippocampus. This agrees with reports of GCs or stress (in a GC-dependent manner) increasing levels of TNF- α , IL-6, and IL-1 β in the periphery and the brain (for review, see Sorrells et al., 2009).

In the frontal cortex, mid-CORT levels had the opposite effect of low or high levels, potentiating proinflammatory gene expression induced by LPS. The existence of two types of receptors for GCs, namely the high-affinity mineralocorticoid receptor (MR), and the lower affinity GR, could explain this pattern. MR is heavily occupied basally and becomes saturated by GC levels in the mild stress range, whereas GR is heavily occupied only after major stressors. MR and GR often have opposite effects (de Kloet, 2000). In our experimental model, GR is not heavily occupied until the GC range of major stressors (i.e., 30% pellets or higher) (Stein-Behrens et al., 1994); this agrees with our prior work showing GR mediation of the proinflammatory effects of GC on LPS-induced NF- κ B activation and gene expression (Munhoz et al., 2006). While concentrations of GR are similar in the frontal cortex and hippocampus of rats, MR is expressed more in the latter (Joëls et al., 2008). The proinflammatory GC effects also occurred in the hippocampus, producing an inverse-U pattern

Figure 6. Influence of CORT levels on phosphorylated forms of MAP kinases 42/44 MAP kinase (**A**), p38 MAP kinase (**B**), SAPK/JNK (**C**), and AKT (**D**) in the absence (SAL) or presence of LPS in the frontal cortex. Top, Representatives of Western blot autoradiographies. Bottom, Densitometric analysis of the specific bands of SAL- or LPS-treated groups presented in the top panels (mean $\pm$ SEM; $n = 5$ animals per group). Protein levels are presented as ratios of specific kinases to β -actin levels. Bonferroni test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

on LPS-induced expression of the two proinflammatory genes (TNF- α and IL-1 β).

In the frontal cortex, neither low nor high CORT levels increased expression of I κ B- α or MKP-1, an important phosphatase that accounts for some of the anti-inflammatory effects of these hormones.

CORT retains its anti-inflammatory effects after LPS injection

Amid GCs failing to have anti-, and even having proinflammatory effects, they had some classical anti-inflammatory functions. In the frontal cortex, high CORT decreased LPS-induced NF- κ B activation and, in the absence of LPS, increased expression of the anti-inflammatory genes I κ B- α and MKP-1. In agreement with this, although CORT potentiated excitotoxin-induced infiltration of inflammatory cells into the hippocampus, it inhibited it in the absence of an insult (Dinkel et al., 2003).

Furthermore, 15% CORT pellets produced a proinflammatory response in NF- κ B activation, levels of proinflammatory genes (i.e., IL-1 β) and some kinases' activation (i.e., SAPK/JNK) in the hippocampus. It is well known that LPS and cytokines activate the hypothalamus-pituitary-adrenal (HPA) axis and increase GC concentrations. This activation has been suggested to constitute negative feedback regulation due to the immunosuppressive actions of GCs; in this view, these GC actions prevent the immune system of overactivating into a dangerous range (Munck et al., 1984; Beishuizen and Thijs, 2003). These results could be further evidence of anti-inflammatory GCs effects and of the

importance of a functional HPA axis following an inflammatory stimulus.

In addition, another important anti-inflammatory GC effect is to increase the expression of anti-inflammatory genes such as I κ B- α , IL-1 α , and MKP-1 expression. In accord with this, increasing levels of CORT increased LPS-induced expression of these genes in the hippocampus. In addition, proinflammatory GC effects in the cortex can occur alongside anti-inflammatory actions in the hypothalamus and heart (Munhoz et al., 2006), perhaps reflecting regional differences in GR/MR abundance and function.

Elevated CORT overactivates MAPK pathway after LPS injection

To better understand these proinflammatory CORT effects, we analyzed MAPK and AKT phosphorylation. The MAPK superfamily includes extracellular-regulated kinases (ERKs, also known as p42/p44), c-Jun-N-terminal kinases (JNKs), stress-activated protein kinases (SAPK), and p38-MAPK (Karin, 1998); these kinases are activated by LPS-TLR4 binding, leading to NF- κ B activation (Walton et al., 1998).

In contrast, Pi-3kinase/AKT may decrease inflammation by inhibiting TLR-4 signaling in macrophages (Zhang and Daynes, 2007a). In agreement with this, the AKT pathway decreases neuron death after stroke (Zhao et al., 2006). We

showed that LPS-induced AKT phosphorylation is not augmented by CORT, perhaps reflecting a ceiling effect.

Increasing CORT levels progressively increased ERK1/2, JNK, and p38 kinase activation in frontal cortex and hippocampus in the absence of LPS. Similarly, a variety of stressors induce PKB/AKT and ERK1/2 phosphorylation and activation in the striatum, hypothalamus, cortex, and hippocampus (Lee et al., 2006).

In the presence of LPS, ERK phosphorylation, showed the same pattern observed for NF- κ B activation and proinflammatory gene expression, with an inverse-U pattern in the cortex, and both moderate and high CORT levels showing the same pattern in the hippocampus.

In agreement with our data, intracortical LPS injection with or without stress (via release of cytokines such as TNF- α and IL-1 β) increases phosphorylation of JNK, p38, and ERK (de Pablos et al., 2006), with the stress component being GR-mediated. In addition, low-dose GC conditioning during the differentiation of murine bone marrow-derived macrophages (BMMs) led to a hyper-responsiveness to endotoxins, perhaps due to enhanced LPS-induced activation of NF- κ B and MAPK signaling (Zhang and Daynes, 2007b). We cannot establish whether these changes in kinase activation are a cause or consequence of the GC proinflammatory effects, or the role of each individual kinase in these GC effects.

GCs not only act genomically, but also via cross-talk with other signaling pathways and transcription factors (Schoneveld et al., 2004). GCs counter-regulate MAPK signaling pathways, in particular p38, JNK, and ERK pathways by inducing expression

of MAP kinase phosphatase-1 (MKP-1). MKP-1, a dual specificity phosphatase induced by cellular stress, serum, and growth factors, dephosphorylates and inactivates MAP kinases such as JNK, p38, and ERK1/2 (Clark and Lasa, 2003). GCs induce sustained expression of MKP-1 in HeLa cells (Lasa et al., 2002), osteoblasts (Engelbrecht et al., 2003), macrophages (Roger et al., 2005), and epithelial and endothelial cells (Imasato et al., 2002; Fürst et al., 2007). MKP-1 is also expressed in cultured microglia (Eljaschewitsch et al., 2006). In addition to GCs, proinflammatory stimuli, such as LPS, induce MKP-1 expression, creating a negative feedback loop that downregulates production of TNF- α , IL-1 β , and IL-6 (Shepherd et al., 2004; Zhao et al., 2005). Furthermore, the GR agonist dexamethasone inhibits MCP-1 production and microglial cell migration to an inflammatory site by up-regulating MKP-1 expression and decreasing p38 and JNK MAPK activity (Zhou et al., 2007). Moreover, dexamethasone inhibits p38 kinase activation and induces MKP-1 expression in macrophages from wild-type but not in macrophage from GR-deficient mice (MGRKO) and inhibition of that kinase rescues MGRKO mice from LPS-induced lethality (Bhattacharyya et al., 2007). Here, we showed that MKP-1 expression is decreased in the cortex and increased in the hippocampus of rats that received LPS. This result could indicate that the cortex is more susceptible to GC proinflammatory effects than the hippocampus, and whether MKP-1 is important to this effect, it could be suggesting that the negative feedback loop that down-regulates the production of proinflammatory cytokines is not activated in the cortex.

Cyclin-dependent kinase (Cdk) and MAPK (ERK, JNK, and p38) alter GR function via phosphorylation (Rogatsky et al., 1998). MAPK phosphorylation may influence GR's nuclear transport or affinity to cofactors. Such activation of MAPK pathways not only leads to the phosphorylation and increased transcriptional activity of mitogenic and proinflammatory regulators, including AP-1, NF- κ B and cytokines, but also ensures decreased transcriptional activity of anti-inflammatory and anti-proliferative factors such as GR (Chen et al., 2008). Similarly, maximal NF- κ B activity requires protein–protein interaction and posttranslational modifications (Yamamoto and Gaynor, 2004). In addition, MAP kinase can phosphorylate p65, which is essential for its transcriptional activity (Schmitz et al., 2001; Vermeulen et al., 2003).

There are cell type-specific effects to such GC signaling, with increased TLR-4 and NF- κ B signaling being protective in neurons (Camandola and Mattson, 2007), and damaging in microglia (Kreutzberg, 1996). Stress-induced sensitization of microglial reactivity (“priming” stress effects) enhances inflammation (Frank et al., 2007). Furthermore, stress, of a similar duration as the time course in the present study, induces GC-dependent microglia proliferation (Nair and Bonneau, 2006).

Figure 7. Influence of CORT levels on phosphorylated forms of MAP kinases 42/44 MAP kinase (**A**), p38 MAP kinase (**B**), SAPK/JNK (**C**), and AKT (**D**) in the absence (SAL) or presence of LPS in the hippocampus. Top, Representative of Western blot photographs. Bottom, Densitometric analysis of the specific bands of SAL- or LPS-treated groups presented in the top (mean $\pm$ SEM; $n = 5$ animals per group). Protein levels are presented as ratios of specific kinases to β -actin levels. Bonferroni test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 2. Summary of the main CORT effects on frontal cortex (top) and hippocampus (bottom) of rats treated with saline (SAL) or LPS

	SAL				LPS			
	PRO	Mixed	NO	ANTI	PRO	Mixed	NO	ANTI
Cortex								
NF- κ B			✓			✓		
IL-1 β			✓		✓			
TNF- α			✓		✓			
I κ B- α			✓	✓				
IL-1ra			✓				✓	
MKP-1			✓		✓			
42/44 MAPK	✓					✓		
SAPK/JNK	✓							✓
p38 MAPK	✓							✓
AKT				✓			✓	
Hippocampus								
NF- κ B			✓		✓			
IL-1 β			✓		✓			
TNF- α			✓			✓		
I κ B- α			✓	✓				✓
IL-1ra			✓				✓	
MKP-1			✓					✓
42/44 MAPK	✓				✓			
SAPK/JNK	✓					✓		
p38 MAPK	✓				✓			
AKT				✓			✓	

PRO, Proinflammatory effects for all CORT levels; Mixed, pro- and anti-inflammatory effects dose-dependent; NO, no CORT effects in any level; ANTI, anti-inflammatory effects for all CORT levels.

Figure 8. Schematic of the proposed role of high CORT levels on the proinflammatory response triggered by LPS and related to MAP kinase–NF- κ B pathway in rat brain. Normally, LPS binds to TLR4 and activates the MAP kinase pathway, thus activating NF- κ B and increasing expression of proinflammatory cytokines. Basal levels of GCs inhibit this pathway through a number of steps, including (1) GR interacting with the p65 subunit of NF- κ B, (2) increasing the expression of the inhibitory protein I κ B- α , and (3) increasing expression of anti-inflammatory genes such as IL-1ra and MKP-1 (**A**). However, when GC exposure is elevated and prolonged, the hormone increases and potentiates the proinflammatory response related to MAP kinase–NF- κ B pathway (**B**). Straight lines indicate activation and dotted lines indicate inhibition.

The immunosuppressive effects of high-dose GCs prompt their use in clinical medicine. However, this conceptualization is not uniformly correct. For example, while low-dose GC regimes reduce mortality during sepsis (Minnecci et al., 2004), high-dose regimes can worsen mortality, perhaps due to the immunosuppressive GC effects, worsening secondary infections (Klaitman and Almog, 2003; Annane et al., 2006).

In conclusion, GCs not only do not uniformly suppress neuroinflammation, but can even enhance a number of points in the pathways linking LPS exposure to inflammation. Normally, LPS binds to TLR4 and activates the MAPK pathway, thus activating NF- κ B and increasing expression of proinflammatory cytokines, such as IL-1 β and TNF- α (Fig. 8A). Basal levels of GCs inhibit this pathway via GR interacting with the p65 subunit of NF- κ B, and increasing expression of anti-inflammatory I κ B- α , IL-1ra, and MKP-1. However, when GC exposure is elevated and prolonged, the hormone increases and potentiates the proinflammatory response related to the MAPK–NF- κ B pathway (Fig. 8B).

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