Published in final edited form as:

Brain Behav Immun. 2009 October ; 23(7): 1038–1052. doi:10.1016/j.bbi.2009.06.004.

How T-Cell-Dependent and -Independent Challenges Access the Brain: Vascular and Neural Responses to Bacterial Lipopolysaccharide and Staphylococcal Enterotoxin B

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Abstract

Bacterial lipopolysaccharide (LPS) is widely used to study immune influences on the CNS, and cerebrovascular prostaglandin (PG) synthesis is implicated in mediating LPS influences on some acute phase responses. Other bacterial products, such as staphylococcal enterotoxin B (SEB), impact target tissues differently in that their effects are T-lymphocyte-dependent, yet both LPS and SEB recruit a partially overlapping set of subcortical central autonomic cell groups. We sought to compare neurovascular responses to the two pathogens, and the mechanisms by which they may access the brain. Rats received iv injections of LPS (2 µg/kg), SEB (1 mg/kg) or vehicle and were sacrificed 0.5–3 hr later. Both challenges engaged vascular cells as early 0.5 hr, as evidenced by induced expression of the vascular early response gene (*Verge*), and the immediate-early gene, *NGFI-B*. Cyclooxygenase-2 (*COX-2*) expression was detected in both endothelial and perivascular cells (PVCs) in response to LPS, but only in PVCs of SEB-challenged animals. The non-selective COX inhibitor, indomethacin (1 mg/kg, iv), blocked LPS-induced activation in a subset of central autonomic structures, but failed to alter SEB-driven responses. Liposome mediated ablation of PVCs modulated the CNS response to LPS, did not affect the SEB-induced activational profile. By contrast, disruptions of interoceptive signaling by area postrema lesions or vagotomy (complete or hepatic) markedly attenuated SEB-, but not LPS-, stimulated central activational responses. Despite partial overlap in their neuronal and vascular response profiles, LPS and SEB appear to use distinct mechanisms to access the brain.

Keywords

area postrema; central autonomic system; cyclooxygenase-2; endothelial cells; lipopolysaccharide; perivascular cells; staphylococcal enterotoxin B; vagus nerve

INTRODUCTION

During infection, cytokines released from activated immune cells act on the brain to elicit acute phase responses such as, fever, somnolence, anorexia, lethargy and metabolic effects, including

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Conflict of Interest Statement: All authors declare that there are no conflicts of interest.

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activation of the hypothalamo-pituitary-adrenal (HPA) axis (Dantzer et al., 1998b; Dinarello, 1984, 1988; Elmquist et al., 1997; Krueger et al., 1999). Studies of immune system influences on the central nervous system (CNS) have commonly utilized administration of pathogen analogs such as lipopolysaccharide (LPS or endotoxin), a component of the Gram-negative bacterial cell wall, or cytokines themselves, to model the activation of the innate immune system (Elmquist and Saper, 1996; Elmquist et al., 1996; Ericsson et al., 1997; Rivest, 2001; Sapolsky et al., 1987; Schiltz and Sawchenko, 2002). LPS or cytokines are bound by specific receptors on cells leading to cytokine production and/or release that activate secondary signaling mechanisms to influence the CNS.

Other models have been used to study the CNS impact of activating T-lymphocyte-dependent immune response. These include the use of superantigens, notably staphylococcal enterotoxins A (SEA) and B (SEB) (Proft and Fraser, 2003), which are secreted products of *S. aureus* implicated in food poisoning, toxic-shock syndrome and autoimmune disease (Torres et al., 2001). Superantigens powerfully stimulate clonal expansion of T-lymphocytes bearing appropriate motifs on the variable domain of the β chain of the T-cell receptor in an antigen-independent manner, leading to clonal expansion of targeted T-cells and production of a panel of cytokines largely distinct from those stimulated by LPS (Muller-Alouf et al., 2001).

Because neither SEB nor LPS cross the blood-brain-barrier in appreciable concentrations, the mechanisms by which they influence CNS function have been studied extensively and several routes have been proposed (Elmquist et al., 1997; Schiltz and Sawchenko, 2002; Wang et al., 2004; Watkins et al., 1995). Entry at circumventricular organs, transduction by peripheral nerves, facilitated transport across the barrier, and cytokine-receptor interactions at the brain-fluid interfaces, have all been considered as a basis for such interactions (Dantzer, 1994; Ericsson et al., 1997; Watkins et al., 1995). In the case of LPS, there is substantial evidence to support the view that increased circulating cytokines can be monitored by non-neuronal cells of the cerebral vasculature, namely perivascular and endothelial cells, which engage afferent projections to relevant effector neurons, such as those involved in HPA control, through paracrine effects of locally-released prostaglandins (Ericsson et al., 1997; Scammell et al., 1998; Schiltz and Sawchenko, 2002).

The route(s) employed by SEB to access discrete CNS circuits are unsettled. Previous work has shown that the pattern of cellular activation in the CNS that results from intravenous SEB administration overlaps in part with that induced by LPS (Goehler et al., 2001; Kusnecov et al., 1999; Rossi-George et al., 2005; Serrats and Sawchenko, 2006). Anecdotal observations that SEB challenge can also engage cerebrovascular elements (Serrats and Sawchenko, 2006) could indicate a similar mechanism of CNS accession as employed by IL-1 or LPS (Dantzer, 1994; Elmquist et al., 1996; Elmquist et al., 1997; Ericsson et al., 1997; Sawchenko et al., 2000; Schiltz and Sawchenko, 2002). In contrast, however, it has been reported that CNS responses to intraperitoneal SEB are attenuated by sectioning the abdominal vagus nerve (Wang et al., 2004), or by genetic deletion of tumor necrosis factor- α (TNF α) (Rossi-George et al., 2005). We report here the results of experiments designed to compare and contrast mechanisms used by SEB and LPS to engage CNS response systems, focusing on the cerebral vasculature, circumventricular organs and the vagus nerve as potential sites of transduction. Portions of these data have been presented in abstract form (Serrats and Sawchenko, 2005).

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley albino rats (260–340 g) were used in all experiments. They were housed individually in a temperature-controlled room on a 12:12 hr light/dark cycle with food (Harland Teklan rodent chow 8604) and water freely available. Rats were adapted to handling

for at least 5 days prior to any manipulation. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Salk Institute.

Intravenous (iv) administration of SEB and LPS

Indwelling jugular catheters (PE50) containing sterile heparin-saline (50 U/ml) were implanted (Ericsson et al., 1994; Schiltz and Sawchenko, 2002) under ketamine-xylazine-acepromazine anesthesia (25:5:1 mg/kg, sc). The sealed catheter was positioned with its internal Silastic® (Dow Corning, Midland, MI) tip at the atrium, and was exteriorized at an interscapular position. After 2 days' recovery, awake and freely moving rats were removed briefly from their home cage, injected with 1 mg/kg of SEB (lot numbers 062K4076 and 054K4137; Sigma, St Louis, MO) in 300 µl sterile saline, or vehicle alone. To provide comparisons, groups of similarly prepared rats were injected with 2 µg/kg of lipopolysaccharide (LPS from *E. Coli*, Serotype 055:B55; Sigma, St Louis, MO) or vehicle.

Intravenous pre-treatment with indomethacin

Groups of rats were given intravenous injections of the non-selective cyclooxygenase inhibitor, indomethacin (1 mg/kg or 10 mg/kg), or vehicle (0.04M PBS with 10% ethanol and 0.1% ascorbic acid, pH6.0) through an indwelling jugular catheter (see above). Fifteen min after the intravenous injection of indomethacin or vehicle, LPS (2 µg/kg, iv), SEB (1mg/kg, iv) or saline were administered. Three hours later, the animals were anesthetized and perfused for histology.

Intracerebroventricular injection of clodronate-liposomes

Liposomes were made in our laboratory following the protocol described by N. van Rooijen (van Rooijen and Sanders, 1994). These are polyamellar phosphatidylcholine/cholesterol membranes that encapsulate clodronate (used at a concentration of 250 mg/ml), are mannosylated to facilitate receptor-mediated uptake, and labeled with carbocyanine fluorochrome, DiI, to enable detection of cells that have incorporated them. For icv injection, liposomes are gently shaken to resuspend them and equilibrated to room temperature. Control (encapsulating PBS) or clodronate-liposomes were injected in a volume of 50 µl over 10 min into a lateral ventricle using a 26 ga needle mounted onto stereotaxic arm and attached via PE tubing to a 1000 µl gastight syringe (Bee Stinger, BAS). Rats were allowed 7d to recover prior to testing, the point at which depletion of brain macrophages is maximal, and prior to any substantial repopulation from bone marrow-derived progenitors (Polfliet et al., 2002; van Rooijen and Sanders, 1994). The effectiveness of clodronate liposome treatment was confirmed at the conclusion of the experiment by immunohistochemical labeling for the ED2 macrophage differentiation antigen, a definitive marker for PVCs and meningeal macrophages (see below). Animals were then challenged with intravenous injections of SEB (1 mg/kg), LPS (2 µg/kg) or vehicle as above 1–3 hr prior to perfusion.

Abdominal vagotomy

Rats were anesthetized and submitted to complete subdiaphragmatic vagotomy, selective section of the hepatic branch of the ventral vagal trunk or sham operations. Vagotomy was performed as described (Sawchenko and Gold, 1981) and involved laparotomy, followed by independent identification and sectioning of the major branches (anterior and posterior gastric, hepatic, coeliac, and accessory coeliac) and stripping of the esophagus of both trunks to as near the level of the diaphragm as possible. Hepatic vagotomy involved selective section of the hepatic branch. Sham operations were performed similarly, and the vagal branches were isolated but not severed. At the time of surgery, animals sustaining complete vagotomy and controls received a ring of 6–8 100 nl injections of the retrogradely transported fluorochrome, true blue, into the stomach wall just distal to the level of the gastroesophageal junction, to provide an independent index of the effectiveness of surgery. Postoperatively, rats were offered

wet and palatable foods (in addition to lab chow) until body weight stabilized, typically 7–14 d after surgery. Animals were then reinstated on lab chow until rates of weight gain stabilized, and implanted with jugular cannulae. Two days later, approximately half of each group received intravenous injections of 1 mg/kg of SEB, 2 µg/kg of LPS or vehicle. After 3 hr, they were anesthetized with chloral hydrate and perfused, and their brains were prepared for histology.

Area postrema lesions

A similar two-factor design was used, with separate groups of animals receiving either lesions of the area postrema or sham operations and injections of SEB, LPS or vehicle. Animals were anesthetized and mounted in a stereotaxic device, and the cisternum magnum was exposed after reflection of the atlanto-occipital membrane. Area postrema lesions were performed under visual (microscopic) guidance by aspiration, using a Pasteur pipette drawn to outer and inner diameters of about 0.5 and 0.15 mm, respectively, connected to a vacuum pump drawing 10–12 psi. Sham operations were similar to the point of aspiration. After 2 weeks' recovery, animals were then challenged with intravenous injections of 1 mg/kg SEB, 2 µg/kg LPS or vehicle, and perfused 3 hr later, as described above.

Histology and tissue processing

At time points ranging between 0.5 and 3 hr after injection, rats were anesthetized with chloral hydrate (350 mg/kg, ip) and perfused via the ascending aorta with saline followed by ~700 ml of 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5, at 8°C. The brains were removed, postfixed for 3 hr, and cryoprotected in 15% sucrose in 0.1 M phosphate buffer overnight at 8°C. Five one-in-five series of 30 µm-thick frozen coronal sections through the entire brain were cut and collected in cryoprotectant solution and stored at –20°C until processing.

Immunohistochemistry

Fos protein was detected using a rabbit polyclonal antiserum raised against the synthetic peptide, SGFNADYEASSRC, corresponding to 4-17 residues of human Fos protein (Ab-5, Lot 4191-1-1; Oncogene Sciences, Uniondale, NY), used at a 1:10,000 dilution. COX-2-immunoreactivity was detected with an antisera raised in goat against a peptide corresponding to amino acids 584-604 at the C terminus of the rat COX-2 precursor (Santa Cruz Biotechnology) used at a dilution of 1:5000 – 1:10000. Localization of Fos- and COX-2-immunoreactivities (ir) was performed on free-floating sections using a conventional avidin-biotin immunoperoxidase technique (Sawchenko et al., 1990). Endogenous peroxidase was neutralized by treating tissue for 10 min with 0.3% hydrogen peroxide, followed by 8 min exposure to 1% sodium borohydride to reduce free aldehydes. Sections were incubated with diluted primary antiserum at 4°C for 48 hr in PBS with 0.3% Triton X-100 and 2% blocking serum. The primary antiserum was localized using Vectastain Elite reagents (Vector Laboratories), and the reaction product was developed using a nickel-enhanced glucose oxidase method (Shu et al., 1988). Specificity of the antiserum has been evaluated by direct co-labeling for *c-fos* mRNA over a range of challenge conditions (data not shown). Specific staining for each antiserum in experimental and control tissue was abolished by preadsorption overnight at 4°C with 50 µM of the respective synthetic immunogen.

In situ hybridization

Methods for probe synthesis, hybridization, and autoradiographic localization of mRNA signal were adapted from Simmons et al. (1989). *In situ* hybridization was performed using ³⁵S-labeled sense (control) and antisense cRNA probes labeled to similar specific activities. Full length probes for transcripts encoding the immediate-early gene NGFI-B (2.4 kb; from Dr. J. Milbrandt, Washington University; see Milbrandt, 1988), the vascular early response gene

(Verge; 2.9 kb full-length cDNA from Dr. P. Worley, Johns Hopkins University; see Regard et al., 2004) and cyclooxygenase-2 (COX-2; 0.2 kb; Dr. S. Rivest, University of Laval; see Lacroix and Rivest, 1998), were used. Sections were mounted onto poly-L-lysine-coated slides and vacuum dried overnight. They were then post-fixed with 10% paraformaldehyde for 30 minutes at room temperature, digested with 10 µg/ml proteinase K for 30 min at 37°C and acetylated for 10 min. Probes were labeled to specific activities of $1-3 \times 10^9$ dpm/µg and applied to the slides at concentrations of $\sim 10^7$ cpm/ml, overnight at 56°C in a solution containing 50% formamide, 0.3 M NaCl, 10mM Tris, 1mM EDTA, 0.05% tRNA, 10 mM dithiothreitol, 1x Denhardt's solution, and 10% dextran sulfate, after which they were treated with 20 µ/ml of ribonuclease A for 30 min at 37°C and washed in 15mM NaCl/1.5mM sodium citrate with 50% formamide at 70°C. Slides were then dehydrated and exposed to x-ray films (Kodak Biomax MR, Eastman Kodak, Rochester, NY) for 18 hr. They were coated with Kodak NTB-2 liquid emulsion and exposed at 4°C for 3–4 weeks, as determined by the strength of signal on film. Slides were developed with Kodak D-19 and fixed with Kodak rapid fixer.

Combined immuno- and hybridization histochemistry

To phenotype responsive vascular cells, hybridization histochemical localization of inducible markers was combined with immunoperoxidase staining for discrete markers of vascular cell types. PVCs were identified using a monoclonal antibody raised against the ED2 macrophage differentiation antigen, produced by immunization with rat splenocytes (Dijkstra et al., 1985; Serotec; cat. no. MCA342R). The ED2 antigen has been found to conform to a member of the group B scavenger-receptor cysteine-rich family, CD163 (Fabrick et al., 2007). ECs were identified using a monoclonal antibody, RECA-1, produced by immunization with stromal cells from rat lymph node. The antiserum recognizes a surface protein expressed by all rat endothelial cells (Duijvestijn et al., 1992). Combined staining for mRNA and protein involved minor modifications of the constituent protocols described previously (Schiltz and Sawchenko, 2002).

Analysis

Counts of the number of Fos-ir neurons as a function of experimental status were generated for a set of central autonomic cell groups shown previously to be responsive to SEB and/or LPS, including the oval nucleus of the bed nucleus of the stria terminalis, the paraventricular nucleus of the hypothalamus, the central nucleus of the amygdala, the lateral parabrachial nucleus, the nucleus of the solitary tract and the ventrolateral medulla (encompassing the regions of the A1 and C1 catecholamine cell groups) in 3–5 animals per experimental condition. Counts were generated by counting all Fos-ir nuclei in a complete series of sections through the structure(s) of interest, as defined in adjoining series stained for Nissl material and extrapolating estimated counts using the method of Abercrombie (Abercrombie, 1946).

RESULTS

SEB- and LPS-sensitive vasculature elements

To compare vascular responses profiles elicited by LPS and SEB, rats fitted with indwelling venous catheters were challenged with saline (n=3 per time point) or doses of LPS (2 µg/kg; n=4) or SEB (1 mg/kg; n=4) that elicit comparable activational responses from CNS cell groups that they target in common (see Serrats and Sawchenko, 2006, and below), and killed 0.5, 1, 2 or 3 hr later. Series of sections were hybridized for mRNA encoding either NGFI-B, an inducible immediate-early gene that provides a sensitive generic marker of responsive vascular cells, as well as neurons (Chan et al., 1993; Ericsson et al., 1995; Serrats and Sawchenko, 2006), or the endothelial cell-specific vascular early response gene (Verge; Regard et al., 2004). Patterns of neuronal NGFI-B mRNA induction seen following either challenge were similar to those of Fos-ir expression documented in previous reports (Elmqvist and Saper,

1996; Elmquist et al., 1996; Serrats and Sawchenko, 2006) and described below. Both agents also provoked NGFI-B mRNA expression in vascular-associated cells beginning at 1 hr after injection (Fig. 1). Responses to LPS were consistently stronger, and in instances decorated the vascular wall nearly continuously. Previous work indicates that LPS at this dose activates both endothelial and perivascular cells (PVCs; Schiltz and Sawchenko, 2002). SEB-induced NGFI-B mRNA labeling appeared as isolated grain clusters, nearly all of which co-labeled for the ED2 antigen, a marker of PVCs, in double staining experiments.

The endothelial-specific early response gene, *Verge*, was rapidly (within 30 minutes) and robustly upregulated in response to LPS, and to a lesser degree following an SEB challenge (Fig. 1). Dual staining experiments showed that *Verge* mRNA signal conformed tightly to the distribution of the endothelial cell marker, RECA-1-ir (not shown), and never colocalized with the PVC marker, ED2 (Fig. 1).

COX-2 induction

Because of evidence implicating prostaglandins of vascular origin in engaging CNS responses to LPS and IL-1 challenges (Elmquist et al., 1997; Ericsson et al., 1997; Lacroix and Rivest, 1998; Matsumura et al., 1998; Quan et al., 1998; Scammell et al., 1998), we compared the effects of our standard doses of LPS and SEB on COX-2 mRNA and protein expression. As previously reported (Schiltz and Sawchenko, 2002), LPS at 2 μ g/kg produced a prominent induction of COX-2 mRNA and immunoreactivity in the meninges, choroid plexus, and vasculature-associated cells at 1 and 3 hr after injection, respectively, as compared with rare and widely scattered positive labeling seen in saline-treated animals (Fig. 2). Immunopositive vascular elements displayed two distinct morphologies, one of a multipolar type with prominent labeling of cytoplasm and one or more processes, and the other of less intensely stained round profiles. These have been shown by dual staining to label differentially for PVC and endothelial markers, respectively, with the prominence of endothelial labeling varying directly with LPS dose (Schiltz and Sawchenko, 2002).

SEB injection (1 mg/kg) also activated COX-2 mRNA and protein expression with a similar time course. At the mRNA level, responses to this challenge were consistently weaker than those elicited by LPS at 2 μ g/kg. SEB-induced vascular COX-2 expression was seen exclusively in multipolar cells, presumably corresponding to PVCs.

Role of prostaglandins in LPS and SEB signaling

Prostaglandin-dependent mechanisms have been implicated in the LPS-induced activation of cell groups interconnected network of core structures subserving central autonomic and neuroendocrine control (Saper, 1995), including activation of the HPA axis (Ericsson et al., 1997; Schiltz et al., 2002). To determine if this mechanism generalizes to SEB, rats were pretreated with systemic injections of the non-selective cyclooxygenase inhibitor, indomethacin, 15 min prior to intravenous LPS (2 μ g/kg) or SEB (1 mg/kg) challenges. Rats pretreated with the vehicle ($n=3$) used for indomethacin administration and subsequently challenged with LPS displayed very prominent activation of Fos expression in each of a set of interconnected central autonomic cell groups, including the nucleus of the solitary tract (NTS), the ventrolateral medulla (VLM), the lateral parabrachial nucleus (PBL), central nucleus of the amygdala (CeA), the paraventricular nucleus of the hypothalamus (PVH) and the oval nucleus of the bed nucleus of the stria terminalis (BSTov; Fig. 3), fully compatible with previous reports. Among LPS-injected animals, indomethacin pretreatment ($n=4$) produced clear dose-related decrements in the number of Fos-ir neurons counted in the NTS, VLM and PVH, which were statistically significant ($P<0.05-0.001$) at the higher dose of indomethacin employed (10 mg/kg; Figs. 3 and 4). By contrast, pretreatment with the cyclooxygenase inhibitor did not

reliably affect LPS-induced Fos counts in the remaining central autonomic structures examined (PBl, CeA, BSTov; $P_s > 0.1$; Figs. 3 and 4).

Consistent with our previous report, SEB challenge elicited reliable Fos induction in a subset of central autonomic structures (Serrats and Sawchenko, 2006). This included robust activation of the PBl, CeA and BSTov, and a weaker (relative to LPS), though reliable, response in the NTS. Pretreatment with indomethacin ($n=3$) did not significantly alter any of these cell groups' responses to iv SEB challenge ($P_s > 0.1$; Fig. 3).

These findings support a partial, region-specific, prostanoid dependence of central autonomic responses to LPS, but fail to provide any indication of such for SEB-induced cellular activation. Interestingly, responses of the three principal cell groups activated in common by the two immune challenges were unaffected by pretreatment with the COX inhibitor.

Role of perivascular cells in CNS responses to LPS and SEB

PVCs are implicated as sensitive sites of LPS-induced COX-2 expression and prostaglandin production (Schiltz and Sawchenko, 2002). Recent work exploiting their constitutive phagocytic activity to ablate them by intracerebroventricular injection of liposomes encapsulating the bisphosphonate drug, clodronate (Polfliet et al., 2001a), has indicated two distinct roles for this cell type in immune-to-brain signaling (Schiltz and Sawchenko, 2003). In response to an IL-1 challenge, in which PVCs are normally the only detectable locus of vascular COX-2 induction, depletion of PVCs results in attenuated PVH cellular activation and HPA secretory responses, while these responses to LPS are exaggerated, as is endothelial COX-2 expression. The latter result suggests that PVCs normally inhibit endothelial prostanoid production. Since PVCs are the sole source of SEB-induced vascular COX-2 expression, we sought to determine if this restraining influence generalized to an SEB challenge situation.

Rats were pretreated with icv injection of control (PBS-filled, $n=6$) or clodronate liposomes ($n=6$) and challenged intravenously with saline, SEB (1 mg/kg) or LPS (2 μ g/kg) 5–7 days later, the time at which PVC depletion is maximal and preceding repopulation by circulating progenitor cells (Polfliet et al., 2001b). As reported previously (Schiltz and Sawchenko, 2003 and our unpublished data), rats receiving clodronate liposome pretreatment prior to LPS injection displayed reliable increments in the number of COX-2-positive endothelial cells, and of Fos-ir cells in the PVH, relative to controls that receive PBS-filled liposomes (3061 ± 168 Fos-positive cells in PBS-Lips rats versus 5579 ± 356 Fos-positive cells in rats treated with clodronate liposomes; $P < 0.01$).

Control rats killed 3 hr after intravenous injection of SEB (1 mg/kg) showed the expected induction of Fos-ir in the central autonomic areas highlighted previously (PBl, CeA, BSTov), as well as COX-2-ir vascular-associated cells of PVC morphology. While central injection of clodronate liposomes 5 days earlier completely eliminated SEB-induced vascular COX-2 expression it had no apparent (Fig. 5) or measured effects on the number of Fos-ir neurons in normally SEB-responsive central autonomic structures ($P_s > 0.1$). Rats treated with clodronate-liposomes and injected intravenously with saline did not show any increase of Fos-ir or COX-2-ir labeling in comparison with rats injected with PBS-liposomes and intravenous saline (data not shown).

To clarify the apparently differential effect of brain macrophage depletion on LPS- and SEB-induced vascular responsiveness, counts of cells hybridized for NGFI-B mRNA per unit of vessel circumference were carried out in series of sections through matched forebrain and brainstem regions (Table 1). As reported previously (Schiltz and Sawchenko, 2003), macrophage-depleted animals responded more strongly to LPS, with vessels (i.e., endothelial cells) displaying a 2–3-fold increment in the density of NGFI-B positive cells. In the forebrain,

we counted an average of 1.20 ± 0.08 cells per $100 \mu\text{m}$ of vessels in rats injected with PBS-liposomes versus 2.14 ± 0.13 in rats injected with clodronate-containing liposomes. In the brainstem, there were an average of 1.28 ± 0.14 versus 2.98 ± 0.33 NGFI-B mRNA-positive cells per $100 \mu\text{m}$ of vessels in PBS-Lips versus Clod-Lips respectively. Conversely, brains of animals treated with clodronate liposomes prior to 1 mg/kg SEB challenge showed very few, if any, grain clusters indicative of NGFI-B transcripts associated with vessels. These results support an involvement of prostaglandins and of PVCs in CNS responses to insults that target the innate immune system, but fail to provide evidence of such in T-cell-mediated challenges such as that provided by SEB.

Role of area postrema and medial NTS

The area postrema is the circumventricular component of the dorsal vagal complex, which has been regarded as an interface between blood-borne immune signals and CNS (Goehler et al., 2006). The area postrema exhibits constitutive expression of TNF p55 receptor, which is upregulated at this locus in response to LPS (Nadeau and Rivest, 1999). Intravenous SEB or LPS rapidly increase circulating TNF α levels (Serrats and Sawchenko, 2006), and TNF α -antagonism or deficiency can diminish CNS responses to LPS (Fong et al., 1989) or staphylococcal superantigen, SEA (Rossi-George et al., 2005). Since major projections of the area postrema target the NTS and VLM (Cunningham et al., 1994), it is a candidate for mediating SEB and/or LPS effects on the central autonomic system, including facets involved in HPA control.

Aspiration lesions completely ablated the area postrema in four SEB-, three LPS- and three saline-injected animals. The lesions also included variable portions of the underlying subpostrema, and less consistently medial, regions of the NTS (Fig. 6). Groups of sham-lesioned rats ($n=3$ each) provided comparisons. In these, challenge-induced patterns of Fos-ir in central autonomic cell groups were similar to those reported above and elsewhere (Elmqvist and Saper, 1996; Elmqvist et al., 1996; Serrats and Sawchenko, 2006). For quantitative analysis, we focused on two cell groups, the CeA as representative of structures responsive to both insults, and the PVH as an exemplar of LPS-sensitivity (Fig. 7 and 8). Area postrema lesions resulted in a reliable 51% reduction in the number of CeA neurons responding to SEB ($P<0.05$); the residual response nevertheless remained significantly elevated above that of saline-injected controls ($P<0.05$). Similar lesions did not significantly alter CeA activation in response to an LPS challenge ($P>0.1$).

In the PVH, SEB again failed to provoke reliable Fos induction in either sham- or area postrema-lesioned rats ($P>0.1$). Lesions did, however, reduce (by 40%; $P<0.05$), but not eliminate ($P<0.01$ vs saline) LPS induced Fos expression in the PVH.

Vagal involvement

The vagus nerve has been extensively studied as a conduit for conveying immune information from periphery to brain. Abdominal vagotomy can block or attenuate cytokine- or LPS-induced immediate early gene expression and HPA secretory activity, principally when these stressors are injected intraperitoneally (Bluthe et al., 1996; Ek et al., 1998; Fleshner et al., 1998; Goehler et al., 1999; Maier et al., 1998; Watkins et al., 1995; Wiczorek et al., 2005). There is similar evidence for vagal involvement in CNS (Fos) responses to intraperitoneal SEB administration (Wang et al., 2004). Because of this, and because results of area postrema lesions could be attributable to disruption of its vagal input, we compared the effects of vagotomy on CNS responses to SEB versus LPS. We again focused quantitative analyses on the CeA and PVH, and considered only results from animals in which effectiveness of vagal transection was supported by abolition of retrograde labeling by tracer injections in the forestomach (Fig. 6).

Similar to the effects of area postrema lesions, subdiaphragmatic vagotomy resulted in marked (67%; $P < 0.001$) reductions in Fos expression in the CeA stimulated by SEB, to levels that did not differ reliably from those of sham-operated, saline-injected controls ($P > 0.10$; Figs. 8 and 9). By contrast, vagotomy exerted no significant effect on LPS-induced Fos-ir in PVH, or on the PVH response to either challenge ($P_s > 0.1$).

In view of evidence implicating TNF α in mediating CNS responses to staphylococcal enterotoxins (Rossi-George et al., 2005), and the liver as a major site of TNF α production (Tiegs and Gantner, 1996), we repeated these experiments in animals in which the hepatic branch of the anterior (ventral) vagal trunk was severed selectively. This maneuver yielded an overall pattern of results similar to those seen after complete vagotomy (Figs. 8 and 9), with the only reliable effect being a 56% reduction in the response of the CeA to SEB challenge ($P < 0.01$). SEB-induced Fos-ir in the CeA of rats with hepatic vagotomy did not differ from values from sham-operated saline-injected controls ($P > 0.05$). Overall, these results support an involvement of abdominal, and specifically hepatic, vagal mechanisms in driving CNS responses to intravenous SEB, but not LPS, at least under the challenge conditions employed here.

DISCUSSION

LPS and SEB are bacterial products that elicit distinct immune responses, yet the cerebrovascular and central autonomic response profiles they yield upon systemic administration show partial overlap. Despite these commonalities, our findings indicate that LPS and SEB access the brain by distinct mechanisms. In the case of LPS, they support a major role for CNS engagement by a prostaglandin-dependent mechanism involving interplay between vascular cell types. This mechanism applies to components (NTS, VLM, PVH) of a circuit implicated previously in LPS-induced HPA activation, but not to other LPS-responsive central autonomic cell groups (PBL, CeA, BSTov). These latter cell groups are the principal CNS structures activated by SEB, whose effects may be mediated by circumventricular (area postrema) and/or peripheral neural (vagal) transduction mechanisms.

LPS versus SEB

LPS, a component of the cell wall of Gram-negative bacteria, is the prototypic stimulus used to model activation of the innate immune system. It is bound by receptors (CD14, Toll-like receptor 4) expressed at the margins of the CNS where it may activate inflammatory responses directly (Rivest, 2003), and on myeloid cells leading to production and release of cytokines, prominently including IL-1, IL-6 and TNF α (Turnbull and Rivier, 1999). Central consequences of LPS administration include the full range of “sickness behaviors” listed previously (fever, lethargy, anorexia, somnolence) and HPA activation (Dantzer et al., 1998).

Whereas LPS effects are independent of T-lymphocyte involvement (Janeway and Medzhitov, 2002; Nguyen et al., 2002), superantigens like SEB stimulate T-cells in much higher proportion than conventional antigens, by binding class II major histocompatibility complexes of antigen-presenting cells outside of the conventional antigen-binding groove. Such complexes are then recognized by T-cells that express appropriate motifs on the variable domain of the β chain ($V\beta$) of the T-cell receptor (TCR) (Muller-Alouf et al., 2001). This then triggers clonal expansion of the targeted T-cells, leading to production and release of Th1-type cytokines (IL-2, TNF α and interferon- γ) among other immune effectors.

Along with other staphylococcal enterotoxins, SEB is best known as a common cause of food poisoning, and as a mediator of non-menstrual toxic shock syndrome (Schlievert, 1986; Wright and Trott, 1988). SEB is also capable of enhancing specific humoral immune responses (Torres et al., 2002) and of exacerbating autoimmune diseases (Brogan et al., 2004; Schiffenbauer et

al., 1993). Because of its stability, potency to incapacitate and ability to be aerosolized, SEB was developed as a biological warfare agent, and is listed as a Class B bioterrorism threat by the U.S. Centers for Disease Control. The known central effects of SEB are relatively subtle. It does not mimic the array of sickness behaviors elicited by LPS (Kusnecov et al., 1999), though it can provoke a mild fever (Goehler et al., 2001). SEB activates pituitary-adrenal stress hormone secretion (Bette et al., 2003; Kusnecov et al., 1999; Shurin et al., 1997), though its failure in our hands to engage PVH neurons that govern this response (Serrats and Sawchenko, 2006; present findings) leaves the site of action open to question. The most robust behavioral effect attributed to SEB is a capacity to exaggerate the underconsumption of a novel taste substance (neophobia) in a context-dependent manner (Kusnecov et al., 1999; Rossi-George et al., 2004).

Relation to previous studies

The widespread activation of the central autonomic system seen in the present experiments in response to LPS is consistent with a host of previous studies (Elmquist and Saper, 1996; Elmquist et al., 1996; Rivest and Laflamme, 1995). One departure is the fact that LPS-induced activation of the ventrolateral medulla, while reliable, was less pronounced than has been reported previously (e.g., Schiltz and Sawchenko, 2002). Similarly, the pattern of central autonomic activation elicited by SEB, prominent in the BSTov, CeA and PBL, less so in the NTS and essentially lacking from the VLM and PVH, is similar to our previous findings in rat (Serrats and Sawchenko, 2006). This is in keeping with other reports in the literature, with the exception that others have described marked SEB-induced activation of the neurosecretory compartment of the PVH in rat (Goehler et al., 2001; Wang et al., 2004), and the BALB/c mouse line (Bette et al., 2003; Kusnecov et al., 1999). We have considered in detail potential bases for this discrepancy (Serrats and Sawchenko, 2006) and find no simple reconciliation as our essentially negative finding obtained over a range of doses, routes of administration and using independent markers of functional activation. For present purposes, we adopt the view that stress-related neurosecretory neurons of the PVH differ from core SEB-responsive CNS cell groups at least as regards their sensitivity to the challenge.

LPS and SEB can induce septic or toxic shock, whose effects may include sustained high fever, hypotension, breakdown of the blood-brain barrier and multiorgan failure, including liver toxicity (Dantzer et al., 1998a; Dantzer et al., 2008; Dantzer and Wollman, 1998; Dinges et al., 2000; Steiner et al., 2006). The doses employed here were chosen on the basis of prior dose-response studies (Bette et al., 1993; Kusnecov et al., 1999) to mimic the engagement of acute phase responses to systemic infection, while avoiding the global, nonspecific consequences of shock. For example, either LPS or SEB administration, can result in hypotension, which alone is capable of stimulating Fos induction in the central autonomic structures considered here (Chan and Sawchenko, 1994). The dose of LPS employed here is below the level at which hypotensive effects have been reported in rats (Xia and Krukoff, 2001). Similarly, SEB at the dosage level we employed give rise to only a mild ($\sim 0.4^{\circ}\text{C}$) fever (Goehler et al., 2001). We previously found a stable pattern of SEB-induced Fos expression in brain over iv doses ranging from 250 $\mu\text{g/kg}$ to 5mg/kg, with the 1 mg/kg dose employed in the present study, provoking the expected rise in circulating levels of $\text{TNF}\alpha$ and IL-2 (Serrats and Sawchenko, 2006).

Prostaglandin-dependent vascular mechanisms

The present findings are consistent with prior work supporting a prominent role for prostaglandins of vascular origin in driving at least a portion of central autonomic system responses to LPS. PGE_2 levels in brain are increased following systemic LPS injection (Dinarello et al., 1991; Sehic et al., 1996), and a wealth of evidence indicates that central or peripheral blockade of prostanoid synthesis with COX inhibitors can attenuate or abolish

central and peripheral indices of HPA activation normally seen following this challenge (Ericsson et al., 1997; Katsuura et al., 1988; Lacroix and Rivest, 1998; Watanabe et al., 1990). We have implicated projections arising from catecholamine-containing neurons of the NTS and VLM in mediating PVH and HPA responses to IL-1 and LPS (Ericsson et al., 1994; Li et al., 1996; Schiltz and Sawchenko, 2007), and these are the very three cell groups whose responses to LPS in the present study were essentially eliminated by indomethacin pretreatment. Interestingly, LPS-induced engagement of the BSTov, CeA and PBl (the principal triad of SEB-sensitive central autonomic structures) was unaffected by COX blockade or surgical manipulations that disrupted these regions' responses to SEB (see Fig. 7 and below). Despite interconnections between the central autonomic cell groups of interest here (Saper, 1995), they are not necessarily activated *en masse* or by similar mechanisms in response to a given immune challenge. Gaykema and colleagues (2007) highlight the existence of two output pathways from the NTS providing for the distribution of immune-related signals, one arising from non-catecholaminergic NTS neurons and targeting the PBl, and then, in turn, the CeA and BSTov, and a second catecholaminergic pathway involving NTS and VLM projections directly to PVH. The existence of circuits segregated in this way could accommodate our findings implicating separate mechanisms subserving SEB-induced activation patterns (NTS, PBl, CeA, BSTov) and a subset of LPS-responsive ones (NTS, VLM, PVH). Neither mechanism, however, is implicated in the engagement of the BSTov-CEA-PBl subset by an LPS challenge, whose basis remains to be clarified.

The cellular source(s) of vascular prostaglandin production in response to LPS or IL-1 has remained a point of contention, with previous reports being divided as to whether PVCs (Elmqvist et al., 1997; Schiltz and Sawchenko, 2002) or endothelial cells (Cao et al., 1996; Matsumura et al., 1998; Quan et al., 1998) are the dominant seats of vascular COX-2 induction following LPS and/or IL-1 challenges. We offered a reconciliation by identifying PVCs as the sole site of COX-2 expression following IL-1 or low (0.1 µg/kg) doses of LPS, with endothelia recruited in a dose-dependent manner at higher (≥ 2 µg/kg) endotoxin doses (Schiltz and Sawchenko, 2002). The present finding of LPS-sensitive vascular cells identified on morphological and/or phenotypic grounds as including both PVCs and endothelia is consistent with this view.

PVCs are derived from bone marrow precursors that populate the brain postnatally and turn over slowly during adult life (Gehrmann et al., 1995; Thomas, 1999). They are not integral components of the vascular wall, but rather occupy the perivascular space between the endothelial basement membrane and the glial limitans (Gehrmann et al., 1995). They are constitutively phagocytic, and, along with a related cell type in the meninges, constitute the principal brain-resident macrophage populations. This attribute allows these cell types to be selectively ablated by central (icv) injection of liposome-encapsulated drug (clodronate; Polfliet et al., 2001a), a variant of a method used for decades to study peripheral macrophage biology. Recent work using this approach supports a bidirectional interaction between PVCs and endothelial cells transducing LPS or IL-1 signals (Schiltz and Sawchenko, 2003). Thus, in response to an IL-1 challenge that activated multiple markers of vascular PGE₂ production only in PVCs, clodronate liposome pretreatment markedly reduced Fos responses at each level of the NTS + VLM → PVH pathway, as well as pituitary-adrenal hormone secretion. By contrast, each of these responses to LPS (at the same dose as in the present experiments) was exaggerated in macrophage-depleted rats, as were multiple indices of vascular (in this case, purely endothelial) PGE₂ synthesis and release. These findings indicate the dual roles for PVCs as (1) sources of prostanoid production for engaging CNS host-defense systems and (2) in restraining such responses in the vascular endothelium.

Relative to LPS, SEB also provoked a weak endothelial (Verge) response, and more substantial vascular inductions of NGFI-B and COX-2 that were localized to PVCs. Despite this, central

autonomic activational responses to SEB were not reliably affected by indomethacin pretreatment or PVC ablation. In the face of the evidence supporting vascular/prostanoid involvement in the LPS model, basis for these negative results with SEB are not clear. It is possible that the generally weaker vascular response to SEB may generate insufficient prostaglandin production to engage CNS response systems. Alternatively, we have reported that SEB activates cell groups (medial prefrontal cortex, GABAergic hypothalamic neurons; Serrats and Sawchenko, 2006) that have been shown to participate in the inhibitory regulation PVH and HPA output (Boudaba et al., 1996; Figueiredo et al., 2003; Roland and Sawchenko, 1993; Spencer et al., 2005), which could counteract a prostaglandin-based stimulatory drive. Finally, it must be borne in mind that cyclooxygenases catalyze the production of an intermediate (prostaglandin H_2) that may subsequently be processed to yield at least four distinct bioactive prostanoids in addition to PGE_2 (Ivanov and Romanovsky, 2004), and information is lacking as to which of these may be produced by PVCs in response to SEB. This is reinforced by the fact that the terminal PGE_2 synthase to which COX-2 activity is coupled (mPGES-1) has been definitively localized to endothelial cells, while its presence in PVCs is still unsettled (Ek et al., 2001; Engblom et al., 2002; Yamagata et al., 2001). Overall, while vascular COX-2 expression induced by IL-1 or LPS covaries with PVH/HPA activation over a range of experimental conditions, this relationship clearly does not extend to challenges imposed by SEB, at least.

Interceptive signaling via the dorsal vagal complex

Circumventricular organs are widely recognized as portals by which circulating macromolecules, excluded from areas protected by the blood-brain-barrier, can access the brain. While forebrain CVOs were initially considered as sites for transducing circulating immune signals (Blatteis, 1992; Goehler et al., 2006; Rivest, 2003), the area postrema has garnered increasing attention in this regard (Goehler et al., 2006; Lee et al., 1998). Major neural projections of the area postrema target the NTS and PBI (Cunningham et al., 1994), both of which exhibit SEB-sensitivity, with responsive cells in the NTS conforming mainly to non-catecholaminergic neurons (Serrats and Sawchenko, 2006). Because the massive NTS projection to PBI also arises mainly from non-catecholaminergic neurons (Milner et al., 1984), and AP-targeted subregions of the PBI project prominently to SEB-sensitive cell groups in limbic forebrain (Fulwiler and Saper, 1984; Saper, 1995), an AP→NTS→PBI→CeA/BSTov circuit could account for the activational pattern induced by the superantigen. The present findings, in which area postrema ablations attenuated, but did not abolish, SEB-induced Fos responses in each downstream component, support an involvement of such a pathway. Evidence exists to warrant consideration of circulating $TNF\alpha$ in engaging this circuit. SEB provokes a rapid increase in plasma $TNF\alpha$ (Serrats and Sawchenko, 2006), the type 1 $TNF\alpha$ receptor (p55) is prominently expressed in the AP (Nadeau and Rivest, 1999) and that mice deficient in $TNF\alpha$ expression show greatly diminished CNS and behavioral responses to a staphylococcal enterotoxin challenge (Rossi-George et al., 2005). $TNF\alpha$ is also capable of activating neurons in the dorsal vagal complex, and can potentiate glutamatergic signaling by central vagal afferents (Hermann and Rogers, 2008, 2009; Hermann et al., 2003).

The vagus nerve has been widely implicated as a route by which immune signals can access the brain. Section of the abdominal vagus nerves can attenuate or abolish CNS responses to IL-1 or LPS challenges given via the intraperitoneal route, which may be considered as modeling peritonitis (Fleshner et al., 1995; Wan et al., 1994; Watkins et al., 1995; Wiczorek et al., 2005). While vagal sensory neurons may exhibit sensitivity to such agents administered intravenously (Ek et al., 1998), the weight of the evidence indicates that vagotomy does not markedly alter CNS activational responses under these conditions (Ericsson et al., 1997; Katsuura et al., 1988; Romanovsky et al., 1997; Sehic et al., 1996; Wan et al., 1994), and the

present failure of complete or partial vagotomy to affect central autonomic responses to intravenous LPS is consistent with this view.

Vagotomy has also been reported to attenuate intraperitoneal SEB-induced Fos expression in central autonomic cell groups (Wang et al., 2004), and here we extend evidence for vagal involvement to include such responses to SEB given intravenously. We are unaware of another CNS response to *intravenous* immune challenge that depends as heavily on the integrity of the abdominal vagi. The present findings indicate further that selective section of the hepatic branch of the ventral vagal trunk is nearly as effective as complete abdominal vagotomy in mitigating SEB-induced central autonomic activation. This may point to the liver as a critical site in mediating central effects of SEB on the CNS, though it must be noted that fibers traveling in the hepatic branch distribute to other tissues of the upper GI tract as much or more as they do to the liver, per se (Berthoud, 2004). Yet in view of the evidence implicating TNF α as an important mediator of SEB effects on brain, and evidence that T-cell activation by a range of stimuli, including SEB, strongly provoke hepatic TNF α production (Schumann et al., 2000; Tiegs and Gantner, 1996), an hepatic vagal sensory mechanism sensitive (directly or indirectly) to TNF α may warrant consideration as a potential substrate for imparting SEB influences on brain. Vagal sensory neurons are reported to express the p55 TNF α receptor, though immunoreactivity for this receptor has not been detected in peripheral processes of vagal neurons (Hermann et al., 2004).

The relationship between candidate transduction mechanisms in the area postrema and vagus nerves is unclear. On one hand, these could represent separate and complementary avenues by which SEB signals access the brain. Alternatively, because the area postrema is innervated by primary vagal afferents (though not prominently by ones carried in the hepatic branch; Norgren and Smith, 1988), they could represent, in part, separate links in a common pathway.

Finally, it should be noted that while effects of vagotomy on central responses to immune challenge are commonly attributed to disruption of vagal sensory mechanisms, an anti-inflammatory vagal *motor* pathway has been described (Tracey, 2007), and stimulation of the distal end of transected vagus nerves markedly can dramatically reduce LPS-stimulated cytokine responses, including hepatic TNF α production (Borovikova et al., 2000). Thus, while our findings support both circumventricular (area postrema) and peripheral neural (vagal) mechanisms in CNS responses to an SEB challenge, further work is required to ascertain the nature of their involvement and relationship to one another.

Acknowledgments

The authors thank Carlos Arias and Kris Trulock for assistance with surgery/histology and imaging/graphics, respectively. This work was supported by NS-21182, and was conducted in part by the Clayton Medical Research Foundation. PES is a Senior Investigator of the Clayton Medical Research Foundation. JS was supported by fellowships from the Spanish Ministry of Education and Science and the Carlos III Health Institute.

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Figure 1.

LPS- and SEB-induced activational responses in vascular cell types. Darkfield photomicrographs of emulsion-coated slides showing vascular expression of mRNAs encoding the immediate-early gene, NGFI-B (top row), and the vascular early response gene, Verge (bottom row), in rats sacrificed 30–60 min after iv injection of vehicle (left), LPS (middle) or SEB (right). From low levels in controls, both pathogens provoke recruitment of vascular cells, with responses to LPS being more robust. Insets in bottom panels show dual localization of the perivascular cell marker, ED2-ir, with NGFI-B and Verge mRNAs. ED-2-ir perivascular cells (brown) and NGFI-B (top) or Verge (bottom) mRNAs (black grains) around blood vessels. Some NGFI-B mRNA-positive cells manifest ED2-ir (black arrows), but none of the ED2-ir cells (red arrowheads) display Verge mRNA signal (blue arrowheads), indicating a non-macrophage phenotype of Verge-positive cells. Scale bars: 100 μ m.

Figure 2.

LPS- and SEB-induced COX-2 mRNA and protein in vascular cells. Upper panels: Darkfield micrographs of emulsion coated slides showing COX-2 mRNA induced in CNS blood vessels from rats sacrificed 1hr after iv injection of LPS (2 μ g/kg; left) or SEB (1 mg/kg; right). Bottom: Brightfield micrographs showing vascular-associated COX-2-ir from rats sacrificed 3hr after iv LPS (left) or SEB (right) treatment. At doses, LPS (right) elicits enzyme induction in two cell types, a stronger response in multipolar cells (black arrows; previously identified as perivascular cells) and a weaker one in round nuclear cells (red arrows; endothelia). By contrast, SEB (left panel) stimulates COX-2-ir only in multipolar cells (perivascular cells; black arrows). Scale bars: 50 μ m.

Figure 3.

Effect of graded levels of prostaglandin synthesis blockade on LPS- and SEB-stimulated Fos-ir in the PVH and CeA. Brightfield images of sections through similar levels of the PVH (top) and CeA (bottom) stained for Fos-ir from animals pretreated intravenously with vehicle or 1 or 10 mg/kg indomethacin (Indo) 15 min prior to iv challenge with LPS or SEB. Indomethacin pretreatment produces a dose-related decrease in LPS-induced Fos-ir in PVH, but not CeA. By contrast, nonselective COX inhibition affects neither the low level of SEB-induced Fos expression in PVH nor the more robust response in the amygdala. Other abbreviations: CeAm, l, medial, lateral parts of central nucleus of the amygdala; dp, dorsal parvocellular part of PVH; mpd, v, dorsal, ventral medial parvocellular parts of PVH; pm, posterior magnocellular part of PVH. Scale bars: 100 μ m.

Figure 4.

SEB- and LPS-induced activational responses in select CNS regions of normal animals (NB) and of animals treated with 10 mg/kg indomethacin (Indo). Mean $\pm$ SEM number of Fos-ir neurons in the indicated cell group of rats killed 3 hours after iv injection of saline (open bars), 1 mg/kg SEB, or 2 μ g/kg LPS in normal animals (black bars) or animals pre-treated with vehicle (V) or 10 mg/kg indomethacin (gray bars). Pre-treatment with indomethacin markedly reduced LPS-induced Fos-ir in a subset of central autonomic cell groups (PVH, NTS and VLM). By contrast, no reliable effect of cyclooxygenase blockade was detected in any of the regions examined in SEB-treated animals. *, differs significantly from saline-injected controls at $P < 0.05$; **, $P < 0.01$; ***, $p < 0.001$. †, differs reliably from respective vehicle-pretreated controls at $P < 0.05$; ††, $P < 0.01$; †††, $p < 0.001$. n, non-significant ($P > 0.05$).

Figure 5.

Liposome treatment effects on PVH and CeA responses to LPS and SEB. Fos-ir in the PVH and CeA of rats treated with clodronate liposomes and subsequently challenged with intravenous LPS (top and middle panels) or SEB (bottom panel) and killed 3hr later. Relative to control levels (see Fig. 4) clodronate-liposome treatment enhances PVH responses to LPS (note higher density of activated neurons in the magnocellular region; pm), while not affecting that in the CeA. By contrast, liposome-mediated macrophage ablation does not modify SEB-induced responses in CeA or in PVH (not shown). Other abbreviations as in Fig. 3. Scale bars: 100 μ m.

Figure 6.

Confirmation of area postrema lesions and abdominal vagotomy. Top: Brightfield photomicrographs of Nissl-stained sections through the level of area postrema, showing the appearance of the region in a sham-operated control (Intact) and in a rat that underwent aspiration of the area postrema (AP). Bottom: Brightfield photomicrographs showing the robust retrograde labeling (immunopositive signal for True Blue) displayed by vagally intact rats of cells in the dorsal motor nucleus of the vagus (DMX; arrows) resulting from retrograde tracer injections placed in the wall of the stomach at the time of surgery, and the complete lack of such labeling in a rat subjected to complete abdominal vagotomy. Other abbreviations: cc, central canal; NTS, nucleus of the solitary tract; XII, hypoglossal nucleus. Scale bars: 200 μ m.

Figure 7.

Area postrema lesions modify Fos expression in SEB-, but not LPS-responsive central autonomic cell groups. Brightfield photomicrographs showing sections through comparable levels of the PVH (top), CeA (middle) and PBL (bottom) stained for Fos-ir from sham-operated controls (Sham) and animals that sustained aspiration lesions of the area postrema (APX) were subsequently challenged with 1mg/kg SEB (left grouping) or 2 μ g/kg LPS (right). Ablation of the area postrema markedly attenuated SEB-induced Fos-ir in the CeA and PBL, but reduced LPS-induced activational responses only in the PVH. Other abbreviations: CeAl, m, lateral, medial parts of the central nucleus of the amygdala; cl, central lateral parabrachial nucleus; el, external lateral parabrachial nucleus. Scale bars: 100 μ m.

Figure 8.

Lesion effects on SEB- and LPS-induced activation of representative central autonomic cell groups. Mean $\pm$ SEM number of Fos-ir neurons in the CeA and PVH of rats killed 3 hours after iv injection of saline (open bar), 1 mg/kg SEB or 2 μ g/kg LPS in sham operated rats (C; black bars) or animals that underwent area postrema lesions (APX) or complete (VX) or selective hepatic (hVX) vagotomy (gray bars). Each of these manipulations significantly reduced SEB-induced Fos expression in the CeA. The only reliable effect on LPS effects was an attenuation of PVH activation in area postrema lesioned rats. *, differs significantly from saline-injected controls at $P < 0.05$; **, $P < 0.01$; ***, $p < 0.001$. †, differs reliably from respective surgical controls at $P < 0.05$; ††, $P < 0.01$; †††, $p < 0.001$. n, non-significant ($P > 0.05$).

Figure 9.

Vagotomy modifies SEB-, but not LPS-, induced Fos expression in central autonomic cell groups. Top two rows: Brightfield photomicrographs showing sections through comparable levels of the PVH and CeA stained for Fos-ir from animals that underwent sham operations (Sham) or complete subdiaphragmatic vagotomy (VX) (lower two rows) three weeks prior to intravenous challenge with 2 μ g/kg LPS (left) or 1mg/kg SEB (right). Bottom two rows: Arranged similarly, but showing sections through the CeA and BSTov from rats that received sham or selective hepatic vagotomies (hVX) prior to LPS or SEB challenge. Either complete subdiaphragmatic vagotomy or selective hepatic branch vagotomy markedly attenuated SEB-induced Fos-ir in the CeA and BSTov, but did not affect LPS-induced activational responses in either the PVH, CeA or BSTov. Other abbreviations: ac, anterior commissure; ic, internal capsule; lv lateral ventricle. Scale bars: 100 μ m.

Figure 10.

Schematic summary on a mid-sagittal image of rat brain to illustrate circuits and mechanisms implicated as underlying central autonomic activational responses elicited by systemic LPS (red) versus SEB (blue) challenges. The present and previous data support a role for cerebrovascular transduction of LPS signals, with resultant prostanoid synthesis driving engagement of the NTS and VLM, and, consequently, targets of their axonal projections in the hypothalamus (PVH). By contrast, a circumventricular organ (CVO; the area postrema (AP)), and/or peripheral neural (vagal) mechanisms are implicated in driving responses of a distinct subset of central autonomic cell groups (BSTov, CeA, PBI) to SEB. Although these same structures are also responsive to LPS, none of the manipulations attempted here affected their recruitment by LPS, leaving its basis an open question.

Table 1

Density of vascular cells exhibiting activational responses to LPS or SEB in intact and brain macrophage-depleted rats¹

	<u>LPS</u>		<u>SEB</u>	
	<u>PBS-lips</u>	<u>Clod-lips</u>	<u>PBS-lips</u>	<u>Clod-lips</u>
Forebrain	1.20±0.08	2.14±0.13 [*]	0.97±0.14	0.26±0.02 ^{**}
Brainstem	1.28±0.14	2.98±0.33 [*]	0.73±0.08	0.08±0.03 ^{***}

¹ Number of NGFI-B mRNA-positive cells per 100 µm of brain blood vessels in rats that were pre-treated with PBS-filled liposomes (control) or clodronate-containing liposomes and subsequently challenged with LPS (2 µg/kg) or SEB (1 mg/kg) and killed 1 hr later. The number of silver grain clusters representing cells positively hybridized for the immediate-early gene marker were counted in a series of sections through select forebrain and brainstem regions. The circumferences of vessels within the sampled regions were calculated and summed, and values are expressed as cell number per 100 µm vascular perimeter. Clodronate-liposome treatment reliably enhanced the vascular responses to LPS, while suppressing those to SEB.

^{*}, differs significantly from PBS-lips controls at P<0.05;

^{**}, P<0.01;

^{***}, p<0.001.