Central injections of prostaglandin activate the hypothalamus and suppress splenic cytokine production

by

Li Pan

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CENTRAL INJECTIONS OF PROSTAGLANDIN ACTIVATE THE HYPOTHALAMUS AND SUPPRESS SPLENIC CYTOKINE PRODUCTION

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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CONTENTS

ABSTRACT

It has been shown previously that immune cells produce pro-inflammatory cytokines such as TNF-alpha, IL-1 beta and IL-6 which can signal the central nervous system and result in immune suppression mediated by the hypothalamic-pituitary-adrenal system and sympathetic innervation of lymphoid organs such as the spleen. Central injections of the cytokine IL-1β suppress the secretion of IL-1β by splenic macrophage following endotoxin stimulation in vitro and increase norepinephrine (NE) turnover in the spleen. In addition, plasma corticosterone is elevated and T cell proliferation and natural killer (NK) cell activity is suppressed. Since the central activational effects of IL-1 beta, as well as endotoxin, have been shown to be blocked by the cyclooxygenase inhibitor indomethacin, we have tested whether central injections of prostaglandin-2 (PGE2) suppresses the production of splenic cytokine in vivo following an endotoxin challenge. In the first experiment, adult male rats were implanted unilaterally with an intracerebroventricular (ICV) cannula in the lateral ventricle. One week later, rats received ICV injections of a long-acting PGE2 analogy (15-methyl PGE2; 4 μg) in 20 μl, or saline. Thirty min after the ICV injection, all rats received an IV injection of endotoxin (0.1 μg LPS) and were killed one hour later. The spleens were removed and rapidly frozen and the rats were then perfused with fixative. The brains were processed for the immunocytochemical localization of c-fos protein in order to index the central activating effects of ICV PGE2, whereas the production of splenic cytokines following an LPS challenge was quantified by Northern blotting with digoxigenin-labelled riboprobes.

Relative to saline injected controls, PGE2 injections produced a significant and dramatic increase in the number of c-fos protein positive neurons in the paraventricular nuclei (PVN) and supraoptic nuclei (SON) of the hypothalamus. Analysis of splenic levels of TNF-alpha and IL-1 beta mRNA indicated that relative to saline controls, PGE2 produced a significant decrease in TNF-alpha mRNA. However, IL-1 beta mRNA levels were comparable between the PGE2 and saline injected groups.

In the second experiment, the rats were divided into two groups composed of sham surgeries and splenic nerve sections. Rats were given an ICV injection of the PGE2 (4μg in 20 μl saline) and 30 minutes later injected iv with 0.1 μg LPS. It was predicted that cutting the splenic nerve abrogated the immunosuppressive effects of central PGE2 on splenic TNF-alpha mRNA levels. Thus the immune effects of central PGE2 on splenic immune function are mediated by the splenic sympathetic nerve. These results indicate that PGE2 activates central neurons at autonomic and neuroendocine regulatory sites and demonstrates that central injections of PGE2 produces a selective suppression of splenic TNF-alpha mRNA levels following an endotoxin challenge. These results suggust that the sympathetic nervous system plays a central role in mediating this centrally induced suppression of splenic cytokine production.

INTRODUCTION

A functional connection between the central nervous system (CNS) and the immune system has been proposed. The sympathetic innervation of lymphatic organs is well documented (Felten et al, 1987; Nance and Burns, 1987) and lymphocytes express receptors for the sympathetic transmitter, norepinephrine (NE) (Roszman and Carlson 1991). Since the brain possesses receptors for cytokines released by activated immune cells, cytokines may act as neuromodulators (Farrar et al, 1978). The suggestion has been made that the CNS, signaled by a neuromodulator produced by immune cells, provides a restraining influence on the immune response via the hypothalamic-pituitary-adrenal axis (HPA) as well as the sympathetic nervous system (Besedovsky et al, 1979; Sundar et al, 1990; Brown et al, 1991; Madden and Livnat, 1991). Interleukin-1(IL-1), a cytokine produced by macrophages, acts as such a neuromodulator and has been shown to have a number of CNS effects (Besedovsky, del Rey, Sorkin and Dinarello, 1986; Dinarello, 1988; Dinarello, 1991). Both IL-1 and interferon-gamma have been shown to increase the activity of the splenic nerve (Ichijo et al 1994; Katafuchi et al 1993). Central injection of IL-1 beta increased splenic sympathetic nerve activity (SNA) (Ichijo et al 1994) and suppressed splenic natural killer (NK) cytotoxicity and the immunosuppression was partially blocked by pretreatment with a sympathetic ganglion blocker (Sundar et al 1990). Also, the suppression of splenic immune responses (IL-1 and IL-2 production, natural killer activity, and lyphoproliferation) after central injection of IL-1 or interferon-gama was blocked by splenic nerve section or pharmacological blockade of sympathetic activity

(Brown et al 1991, Sundar et al 1990, Take et al 1993). Interesting, the suppression of splenic immune function can also be blocked by pretreatment icv with a cyclooxygenase inhibitor (Ichijo et al 1994). These results provide evidence that the activity of splenic sympathetic nervous system (SNS) signalling from the brain is mediated by PGE2 that is induced in the brain.

An immunomodulating pathway should be activated by naturally occurring infectious agents like bacteria, and their products such as endotoxin (LPS, a toxic element of bacteria). PGE2, one of the many products induced by endotoxin has been demonstrated to impact on brain-immune function. It acts as an intermediate hormonal messenger at the interface between blood and brain (Komaki, Arimura, and Koves, 1992; Rivier, 1991; Tlilders et al, 1994). PGE2 functions as a pivotal inflammatory mediator and may represent a primary signaling pathway for the CNS. Increased production of pro-inflammatory cytokines during a host response to infection or injury is associated with alterations in CNS function, such as fever, which is mediated by neuronal activity in the anterior and preoptic area of the hypothalamus (Stitt and Shimada, 1989; Thomas, 1996). Furthermore, PGE2 administered into the brain increases the activity of the HPA axis (Watanabe, Morimoto, Sakata and Murakami, 1990) and the sympathetic nervous system (Feurerstein et al 1982; Ando et al 1995; McNeil et al 1997).

The following sections of this thesis will review the evidence for brain-immune interactions, a functional sympathetic brain-immune pathway, the role of the cytokines IL-1-beta and TNF-α in neuroimmunoregulation, and the application of c-fos immunocytochemistry. This will be followed by an overview of neuronal regulation by

PGE2 and summarize the information available on the involvement of PGE2 in the immune system. Also reviewed will be some autocrine/paracrine interactions between PGE2 and inflammatory mediators as well as discuss the potential role and mechanism of action of PGE2 on some diseases and on the neuro-immune regulatory system.

Brain-immune pathway

Brain to immune signalling

Research on brain-immune interaction has sought to demonstrate a direct action of the brain on peripheral immunity. One approach has been to demonstrate a relationship between psychological state and immune responsiveness. This has ranged from clinical studies seeking a connection between depression and lymphocyte function (Wodaz et al, 1991), the effects of examination stress on Epstein-Barr antibody levels (Kiecolt-Glazer et al, 1986), and the effects of exposing rats or mice to various forms of emotion provoking stimuli on immune competence (Croiset et al, 1990). Also, stressors such as footshock, active avoidance and restraint have been utilized to induce changes in immune function in animals (Keller, Schleifer and Demetrikopoulos, 1991; Berkenbosch, Wolvers and Derijk, 1991).

Another approach to demonstrating that a signal from the brain could affect peripheral immunity has been to condition immune responses. Conditioning implies an associative process occurring in the brain such that a cue paired with a known immune modulator becomes capable of inducing the immune effect when presented alone to the

animal (Ader and Cohen 1985; Spector 1987). An audiovisual cue which had been paired with an antigen has been shown to elicit an allergic response when presented alone to rats (MacQueen et al, 1989). An odor cue previously paired with chemotherapy has been shown to inhibit tumor growth (Ghanta et al, 1990) and conditioned tolerance was observed when an odor was paired with an immunostimulatory drug (Dyck and Greenberg 1991). Using a taste aversion paradigm in which saccharine was paired with an immunosuppressive drug, an immune suppression was observed upon re-exposure to saccharine (Kusnicov, Husband and King, 1988). Stress and emotion provoking stimuli have been paired with cues and have been shown to alter immune function following re-exposure to the cue (Zalcman et al 1989; Lysle et al 1990).

Brain-immune Neuro-pathways

In parallel with these psychologically based studies, a number of investigators have examined the neural mechanisms by which a signal originating in the brain could affect immunity. In that line of research, brain areas such as the septum and hippocampus have been lesioned (Nance et al, 1987). Lesions of the hypothalamus, limbic system, brainstem autonomic nuclei and cortex were shown to result in altered immune function (Reviewed in Felten et al, 1991). Recently, septal lesions have been shown to suppress levels of splenic macrophage production of TNF alpha (Wetmore et al, 1993). However, the signaling pathway from brain to immune system has not been clearly defined. Stress research has shown that glucocorticoids are immunosuppressive and an HPA mediated pathway has been proposed (Bateman et al, 1989). This model pre-supposes that

communication between the brain and immune system is bi-directional. That is, activated immune cells release factors which are capable of signaling the brain (Blalock, 1989) and the brain has been shown to respond to immunization by changes in neuronal firing rates (Besedovsky et al, 1977; Saphier et al, 1987). In addition, changes in NE content and turnover were observed in key brain areas such as the hippocampus (Carlson et al, 1987) and hypothalamus (Kabiersch et al, 1988), the latter apparently induced by IL-1.

Immune to Brain signallging

Considerable evidence has accumulated that the cytokine IL-1 is released by activated macrophages and results in activation of the HPA and an increase in circulating corticosterone followed by immune suppression (Besedovsky, 1979, Besedovsky et al, 1986) Besedovsky and del Rey, 1987). However, there is evidence of another pathway. Conclusive evidence of sympathetic innervation of the lymphoid organs has been demonstrated (Feline et al 1987; Nance et al 1987; Nance and Burns 1989). In addition, splenic denervation or chemical sympathectomy resulted in an enhanced immune response in the spleen as measured by the number of plaque-forming cells (Besedovsky et al, 1979). As well, beta adrenergic antagonists attenuated shock induced suppression of splenic lymphocyte proliferation upon mitogen exposure whereas adrenalectomy did not (Cunnick 1988). In addition, IP injections of IL-1 have been shown to selectively increase NE turnover in the spleen and lung (Akiyoshi, Shimuzu and Saito, 1990). Further studies have shown that the in vitro mitogenic response of splenic lymphoytes can be inhibited by catecholamine agonist and immune cells have been demonstrated to express receptors for

sympathetic transmitters and to respond to transmitter binding with altered function (Roszman and Caarlson, 1991; Heilig et al, 1993). Recently, splenic nerve section was shown to abrogate the effects of footshock on splenic immune function (Wan et al 1993, 1994).

In a series of studies bearing directly on this proposal, a small dose (5 ng) of IL-1 was infused in the lateral ventricles (ICV) resulting in suppression of the splenic T cell proliferation response, natural killer cell (NK) activity (Sundar et al, 1989) and splenic macrophage secretion of IL-1 in response to LPS (Brown et al, 1991). Splenic sympathetic nerve cuts blocked the suppression of splenic macrophages induced by ICV IL-1 (Brown et al, 1991). Similarily, adminstration of a sympathetic ganglion blocker attenuated the suppression of NK activity and splenic T cell mitogen responsiveness (Sundar et al, 1989). In both studies, this sympathetic moduation of splenic immune function was independent of the adrenal glands. Recently, we reported the ICV IL-1 significantly increased the turnover rote of NE in the spleen during the period of immune suppression (Vriend et al 1993). Collectively, these data provide robust evidence for an immunosuppressive sympathetic pathway between the CNS and the spleen which can be activated by the central administration of IL-1. IL-1 is of particular interest in theses studies because of its key role in immune responses and extensive CNS effects which make it a likely neuroimmunomodulator.

IL-1 beta

IL-1 beta is a 17 kd peptide produced by activated immune cells and It mediates the acute phase response to infection. This response includes hepatic acute phase protein synthesis, muscle proteolysis (Martin and Resch, 1988; Sipe, 1985), increases in circulating neutrophils, and such central effects as fever induction, increased slow wave and non-REM sleep (induction sleep), altered hypothalamic norepinephrine turnover and release of the neuropeptides and neuromodulators such as corticotrophin releasing factor (CRF), ACTH, glucocorticoids, vasopressin, somatostatin and alpha-MSH (Besedovsky et al, 1986, 1987), insulin, growth hormone and thyroxin (Kushner, 1982) and prostaglandin E2 (PGE2). In addition, in concert with other cytokines, IL-1 also reduces serum iron and zinc.

IL-1 is present in two forms, alpha and beta which, though they share little homology, appear to bind to the same receptors and have similar physiological effects. IL-1 alpha is generally a membrane bound form, while IL-1 beta is freely secreted and appears to act mainly in a paracrine fashion (Dinarello, 1984; Dinarello, 1991).

IL-1 is reported to be synthesized and released in the brain (Bandtlaw et al, 1990; Higgins and Olschowka, 1991; Minami et al, 1991). In the rat, immunoreactive IL-1 beta has been found in the hippocampus and the hypothalamus, which the most prominent staining being in the hippocampus. Specifically, neuronal processes and terminals were found extending from the hilus of the dentate gyrus into the stratum lucidum and closely associated with the apical dendrites of the pyramidal cells in the CA3 and CA4 fields. Staining was also found in the basal dendrites of the pyramidal cells of the CA3. In the hypothalamus, IL-1 beta was present in the neurons of the paraventricular nucleus with

beaded axons running through the lateral hypothalamus toward the medial basal hypothalamus. Positive fibers terminated in the suprachiasmatic nucleus, basal dorsomedial arcuate, internal and external zones of the median eminence and the posterior pituitary. Fibers were also seen in the periventricular nucleus and the parvocellular area of the paraventricular nucleus bordering the third ventricle and the bed nucleus of the stria terminals (Lechan et al, 1990). In addition to the hippocampus and hypothalamus, IL-1 was found in the basal forebrain and the olfactory bulbs (Lechan et al, 1990). In the human, IL-1 has been found throughout the hypothalamus and the paraventricular nucleus of the thalamus. In addition, cell bodies have been reported near the anteroventral tip of the third ventricle (Breder, Dinarello and Sapier, 1988).

Following intracerebral injection of IFN-r and LPS in the rat, IL-1 beta mRNA was found at the point of injection and also within perivascular cells in the dorsal recess of the third ventricle ventral to the hippocampus and choroid plexus (Higgins and Olschowka, 1991). These were identified as cells of CNS origin and not infiltrating macrophages. This cytokine has been shown to bind extensively in the rat brain with very high densities of receptors found in the densely packed neuronal cell layers of the hippocampus and high densities in the cerebral cortex, the pyriform and cingulate cortex, the anterior dorsal thalamus and the ventromedial hypothalamus (Farrar et al, 1987).

As an interleukin, the primary effect of IL-1 is initiation and expansion of immune reaction. IL-1 beta is produced by macrophages which are the cells which first encounter an immune challenge and are responsible for antigen presentation to T cells. The immune amplifying effect of IL-1 is through induction of IL-2, a T cell growth factor which results

in T cell proliferation and clonal expansion. IL-1 enhances T cell maturation, B-cell activation / proliferation as well as in increase of NK activity which kills virus-infected cells and tumor cells. IL-1 is mainly secreted by peripheral blood monocytes and by pulmonary, peritoneal, splenic, hepatic (Kupffer cells), synovial, and bone marrow's macrophages. Of significance to the present proposal, IL-1 is expressed in the brain by astrocytes, ameboid microglia and by infiltrating macrophages, and plays a role in recovery from brain injury (Dinarello, 1984, 1991; Guilian et al, 1986). As a result of its CNS effects, as well as its presence and binding in the brain, IL-1 may be considered a neurokine and it has been proposed that IL-1 acts as a neuro-immuno-transmitter in the bi-directional communication between the brain and the immune system (Blalock, 1989)

The hypothesis that the central effects of cytokines influence immune function has been tested by Sundar (1989), Weiss (1989) and our laboratory (Brown et al, 1990; 1991). These data demonstrate that ICV injections of IL-1 activate two immunosuppressive pathways: neuro-humoral, mediated by pituitary ACTH which releases circulating glucocorticoids, and a neural sympathetic signal which can affect splenic immune function. The latter is supported by the studies that document the sympathetic innervation of the spleen (Nance and Burns, 1989) and nerve contacts with immune cells (Felten et al, 1987). NE can suppress IL-1 production in splenic macrophages and NE content of the spleen decreases during immunization at the exponential phase of the immune response to SRBC (Green-Johnson et al, 1996; Koff et al, 1986; Del Rey et al, 1981; Besedovsky et al, 1979). Spleen NE turnover is increased during antigen induced increases in sympathetic activity (Fuchs et al, 1988) and following

ICV IL-1 injection (Vriend et al, 1993). NE can change migration of lymphocytes in the spleen and it can stimulate or inhibit immune activity as a function of cell type, adrenoreceptor subtype and temporal parameters (Madden et al, 1989). Sympathectomy is reported to alter T cell activity and antigen presentation in macrophage (Besedovsky et al, 1978). Sympathetic fibers generally contain two types of synaptic vesicles containing classical transmitters and neuropeptides and the peptides may enhance or inhibit transmitter activity (Lundberg et al, 1989). Our demonstration (Nance et al, 1987), and Wetmore et al (1991, 1994) have all found that forebrain areas providing direct or indirect input to the hypothalamus, such as the septal and hippocampal areas, are implicated in the control of immune function. These same areas are targets for IL-1 (Farrar et al, 1987) and corticosterone feedback (McEwen et al, 1969). While IL-1 may not cross the blood brain barrier, there are areas close to the hypothalamus where IL-1 may cross and autoinduce further production of IL-1 or an intermediate which can act as a transmitter. In man, IL-1 immunoreactive fibers innervate the hypothalamus, it is present in cell bodies in the hypothalamus and IL-1 receptors are also located in the hypothalamus (Breder et al, 1988; Farrar et al, 1987). A similar cytokine neural circuit has been described for TNF-alpha in mice (Breder et al, 1993).

TNF-alpha

Tumor necrosis factor (TNF) is a 17 kd polypeptide mediator of inflammation and cellular immune responses (Old 1985; Le and Vilcek 1987; Beutler and Cerami 1988).

TNF is but one member of a family of structurally related cytokines, including lymphotoxin alpha (also known LT alpha or TNF-beta), lymphotoxin beta (LT beta), the fas ligand, and the ligand for the CD40 receptor. TNF is released first in the early stages of immune response by activated macrophages. It was initially described as a cytotoxic molecule which in large doses causes hemorragic necrosis of tumors in vivo and has been shown to be identical with cachectin, a protein which causes the metabolic derangement leading to shock and the cachexia observed during severe disease (Ghiara, Boraschi, Nencioni, Ghezzi and Tagliabue, 1987). In smaller amounts it has been shown to interact synergistically with IL-1 in response to the same immune stimuli such as LPS. In many ways the name tumor necrosis factor is a misnomer, as TNF is generally not toxic to normal cells in vitro. This contrasts with TNF effects on tumor cells, which vary from apoptosis to no effect (Beutler and Cerami. 1989).

The cellular effects of TNF include:

1. In monocytes/macrophages: activation and differentiation, transmigration and chemotaxis (Wang et al, 1990).

2. In vascular endothelial cells: induction of proto-oncogenes (ie, c-fos, c-jun), enzymes (ie, Mn-SOD, collagenase) and integral membrane proteins (ie, adhesion molecules) (Cotran and Pober, 1989), enhance of release of paracrine mediators (ie, PGE2, nitric oxide, endothelin-1, PAF, prostacyclin), modulation of angiogenesis, increase of permeability and enhancement of expression of MHC class 1.

In addition, TNF-alpha causes cytotoxic lymphocyte differentiation, and as a thymocyte comitogen, can induce IL-8 during inflammatory responses. The TNF-alpha

produced by activated macrophages in turn induces IFN-gamma production by NK cells. Subsequently, TNF-alpha and IFN-gamma stimulate IL-2 production and activate macrophage Ia expression and endow tumoricidal activity (Bonavida, 1992).

Recent evidence suggests that the cytotoxic activity of TNF is mediated through p55 receptor (Collart et al, 1990). In some but not all cell types, TNF induces DNA fragmentation as one of the characteristic events of apoptosis. The Fas antigen, also a member of the nerve growth factor/TNF-R superfamily, can signal programmed cell death very similar to that mediated by TNF (Itoh et al, 1991).

TNF receptors are present on active T cell and all cells, except erythrocytes. They are subdivided into two types: type 1 (it is ubiquitous), type 2 (it is restricted to cells of hematopoietic origin). The extracellular domains of both TNF receptors are 27% homologous and significantly related to the nerve growth factor receptor as well as Ox 40, CD40, Fas, and CD27 (Johnson et al 1986). Both subtypes of TNF receptors are present in the serum and urine of patients with febrile illness, sepsis and cancer (Digel et al, 1992; Kalinkovich et al, 1992). These soluble proteins are produced by proteolytic cleavage of the extracellular domain of the TNF-R. Both bind TNF and compete with membrane-bound TNF receptors. At high concentrations, soluble TNF receptors inhibit the bioactivity of TNF. Paradoxically, at low concentrations, soluble receptors appear to stabilize the trimeric "viral particle-like" structure of TNF, slowing spontaneous dissociation and thus increasing the bioavailability of TNF (Aderka et al, 1992). The presence of TNF binding proteins in serum may further serve to deliver TNF to distant sites beyond the microenvironment. Healthy subjects have circulating levels of soluble

TNF-R, which increase markedly and for a prolonged duration after a single injection of LPS (Spinas et al, 1992).

TNF is self inductive, same as IL-1, and both cytokines are mutually inductive. Like IL-1, TNF-alpha is responsible for the induction of acute phase proteins (Last-Barney et al 1988), stimulates the HPA, acts as an endogenous pyrogen, regulates cell metabolism and acts as a growth factor. The main difference between the effects of IL-1 and TNF-alpha is that the latter does not have a direct effect on activation of lymphocytes, although it increases expression of HLA and IL-2 receptors on IL-2 dependent lymphocytes. Both cytokines are inhibited by prostaglandin, corticosteroids, IL-4, IL-6 and tumor growth factor-beta (Rosenblem and Donato, 1989). Despite its overlapping effects with IL-1, its synergistic activity with IL-1 indicates that TNF-alpha and IL-1 have different means of signaling cells. It may be that both cytokines are necessary for the full biological effect during an immune response (Neta, Sayers and Oppenhiem, 1992).

Fever is produced by TNF, IL-1 and many inflammatory mediators and not, as often implied, by IL-1 alone. LPS stimulation of monocytes and macrophages can releases not only IL-1, but TNF-alpha, interferon (IFN-alpha and gamma) and IL-6, structurally distinct polypeptides, all of which induce fever (LeMay et al 1990; Ackerman et al, 1984; Dinarello, 1984; Nakamur et al, 1988; Ater et al, 1985; Billiau, 1981; Bocci, 1980). Evidence for the pyrogenicity of TNF is based on data showing that induction of a fever indistinguishable from IL-1 induced fever follows TNF injection (Dinarello et al, 1986; Kettelhut and Goldberg, 1988; Morimoto et al, 1989; Nakamura et al, 1988; Warren et al, 1987) and that plasma levels of TNF are elevated after LPS administration (LeMay et al,

1990; Michie et al, 1988; Ohaga et al, 1991; Waage 1987). When antiserum to TNF is injected prior to LPS administration, fever is heightened rather than diminished (Long et al, 1990). The reason for this paradoxical effect is unclear.

TNF-alpha enhances the synthesis of IL-1 (Neta et al, 1992; Bachwich et al, 1986; Dinarelo et al, 1986) as well as the synthesis of IL-6 (Shalaby et al, 1989), and IL-6 enhances the synthesis of IL-1 (Helle et al,1988). IFN increases both TNF and IL-1 (Miossec and Ziff, 1986), conversely, they can be inhibited by PGE2, IL-4 and Tumor growth factor-beta (Rosenblum and Donato, 1989). IL-1 increases IL-6 (LeMay et al, 1990; Sirlo et al, 1989) and not suprisingly, antiserum to IL-1 attenuates IL-6, and antiserum to TNF attenuates both IL-1 beta and IL-6 (Fong et al, 1989). Thus multiple cytokines may be necessary for the full biological effect of an immune response to be expresented.

Many of the physiological effects of tumor necrosis factor are comparable to IL-1 (Schobitz, et al., 1994). Similar to IL-1, macrophage secretion of TNF- alpha is under noradrenergic control (Spengler, et al., 1990; Hu, et al., 1991; Spengler, et al., 1994) and therefore likely modulated by the sympathetic nervous system. Macrophage adrenergic receptors (Abrass, et al., 1985) and selective alpha- and beta-agonist have been shown to potentiate and inhibit macrophage cytokine secretion, respectively (Spenler, et al., 1990; Hu, et al., 1991; Severn, et al., 1992; Spengler, et al., 1994; Lgnatowski, and Spengler, 1995) and modify macrophage cytokine mRNA expression (Spengler, et al., 1990). High concentrations of TNF have been implicated in the pathogenesis of numerous disorders (Cush and Lipsky, 1991; Remick and Kunkel, 1989) and the number and the affinity of

beta 2-adrenergic binding sites on mononuclear cells are decreased in these diseases (Baerwald et al, 1992). Agents which act via beta 2-adrenergic receptors have been demonstrated to augment TNF production (Spengler et al, 1990, 1994; Van der Poll et al, 1994; Chou et al, 1996).

C-fos

C-fos, a proto-oncogene, is believed to play a crucial role in biological signaling pathways. The c-fos gene, the normal cellular counterpart of the viral oncogene, v-foc, is expressed at low levels in most cells, but is rapidly and transiently induced in many tissues in response to a variety of physiological and pharmacological agents (Greenberg et al 1985,1986). C-fos encodes a nuclear phosphoprotein (Fos) that exhibits sequence specific DNA binding properties. It has been suggested that Fos may act as a third messenger molecule in signal transduction systems, where it would couple short-term intracellular signals elicited by a variety of extracellular stimuli to long-term responses by altering gene expression (Curran and Franza 1988). These unique characterisitcs have allowed Fos to be used as a selective and reproducible marker to map functional excitatory pathways in the central nervous system (CNS).

Synaptic activity changes postsynaptic gene expression, and the alteration in gene expression induced by neuronal activation can be divided into two general classes. The first consists of immediate early genes (IEGs) whose transcription is activated rapidly and

transiently within minutes of stimulation (Greenberg et al. 1986; Morgan and Cruuan 1986, Bartel et al. 1989, Barzilai et al. 1989). The second consists of late response genes whose expression is induced more slowly and over a longer period of time (hours) (Merlie et al. 1984, Castellucci et al 1988, Goldman et al. 1988, Barzilai et al 1989, Offord and Catterall 1989, Klarsteld et al. 1989). It has been proposed that IEGs encode transcription factors and regulatory proteins that control the expression of late response genes with the late response genes encoding differentiated neuronal products, such as neuropeptides and neurotransmitter biosynthetic enzymes.

Oncogenes were first identified as the genetic products responsible for the induction of tumors by RNA viruses. The proto-oncogene c-fos is the cellular homologue of the viral oncogene V-fos and is expressed in normal cells in vertebrates. The c-fos proto-oncogene was one of the first IEGs identified by Kelly et al (1983), and later confirmed by Greenberg and Ziff (1984). The activation of IEGs by extracellular stimuli has been characterized as not being specific to neuronal cells. The IEGs were originally detected in growth factor-stimulated fibroblasts and this work resulted in the subsequent discovery of IEGs in neuronal cell lines (Curran and Morgan 1987, Lau and Nathans 1987). Although generally expressed at very low or undetected levels in quiescent cells, they are rapidly induced by extracellular stimuli at the transcriptional level. This transcription induction is very transient (30-60 minutes) and independent of new protein synthesis. However, new protein synthesis is required for the subsequent termination of transcription and the mRNAs of IEGs are rapidly degraded (approximately 10-15 minutes in the case of c-fos).

The c-fos gene is the best characterized IEGs at the present time. The c-fos mRNA encodes a protein product, fos protein, to which an antibody can be produced. Fos protein is rapidly synthesized and translocated to the nucleus where it dimerizes with another protein, Jun (or with itself). The heterodimeric protein Fos/Jun activates transcription of genes containing AP-1 sites and regulates the expressions of other genes. Thus Fos immunocytochemestry is a useful marker of functional activity with resolution at the single cell nucleus level (Greenberg and ziff 1984, Kuruijer et al. 1984, Greenberg et al. 1985, 1986, Curran and Morgan 1986, Curran and Franza 1988). In addition to its induction by growth factors, c-fos has been found to be induced by numerous agents, including neurotransmitters (Greenberg et al. 1986, Szekely et al. 1989) and agents that cause an influx of Ca^{++} through voltage dependent Ca^{++} channels (Morgan and Curran 1986). Furthermore, c-fos can also be induced by various kinds of stimuli, including seizures (Morgan and Cohen 1987), hypertonic saline injections (Ceccatelli et al. 1989, Sharp et al. 1991), cutaneous stimulation (Hunt et al. 1987), stressful stimuli (Ceccatelli etal 1989), LPS (Wan et al. 1994) and depolarzing conditions (Morgan and Curran 1986)..

An important role for c-fos in the nervous system was originally demonstrated by several studies performed in the pheochromocytoma cell line (PC12). Transcription of many IEGs, including c-fos, is dramatically induced by electrical stimulation, or exposure to neurotransmitters and growth factors (Greenberg et al. 1985, 1986, Morgan and Curran 1986, Bartel etal 1989). In the presence of nerve growth factor, c-fos transcription is markedly induced by the cholinergic agonist nicotine and by K^{+}-induced depolarization

(Greenerg et al 1985,1986). The Ca^{++} channel agonist BAY K8644 or external Ba^{+} also rapidly induced c-fos in PC12 cells. Subsequently it has been found that c-fos can be induced in neurons by pharmacological (Morgan et al.1987) and physiological stimuli (Hunt et al, 1987, Sagar et al. 1988). The induction of c-fos in response to synaptic activity was first illustrated with convulsant drugs, such as metrazole (Morgan et al 1987, Dargunow and Rebertson 1988, Saffen et al, 1988, Sonnenberg et al. 1989, Watson and Milbranlt 1989) and this response has been detected at both the mRNA and protein level, and can be antagonized by anticonvulsant agents such as diazepam (Morgan et al. 1987) and carbamazepine (Dragunow and Robertson 1987). Inductions of c-fos mRNA also occurs when the motor/sensory cortex is electrically stimulated (Sagar et al. 1988). These initial studies strongly indicated that synaptic activity mediated postsynaptic gene expression. Perhaps most striking induction of c-fos immunoreactivity was observed by Hunt et al.(1987) in spinal cord dorsal horn neurons following peripheral sensory stimulation, and by Sagar et al. (1988) in specific brain nuclei following 24 hours water deprivation. Subsequently, Bullitt (1990) reported that noxious stimuli could induce c-fos in spinal cord neurons and more recently, neurons in the brainstem and spinal cord have been shown to express c-fos protein following treadmill locomotion in cats (Dai et al. 1995) and walking on a rota-rod in rats (Jasmin et al. 1994). The evidence that c-fos can be induced by various stimuli in a broad range of neurons support the claim that c-fos protein is a useful marker of activation of various neurons in the nervous system. However, negative results or the absence of Fos protein production can not be automatically taken to mean that structures or neurons have not been activated by

stimulation. It has been shown that although painful stimuli are very effective at inducting Fos in the dorsal horn of spinal cord , there was little or no Fos expression in the primary afferrent dorsal root ganglia neurons (Menetrey et al. 1989). It has been known that biochemical messengers, including cAMP, calmodulin and G proteins, are required for Fos production (Morgan and Curran 1986, Szekely et al. 1987).

C-fos immunostaining gives single cell resolution and it potentially identifies neurons activated by specific stimuli. It has been demonstrated that electrical stimulation of rat sensory/motor cortex induces fos protein immunostaining in regions known to be connected with motor/ sensor cortex via mono-or polysynaptic pathways, and Fos immunostaining in granule and Purkinje cell nuclei occurred in cerebellar "microzones" which corresponded to parallel results found with 2DG artoradiography (Sagar et al. 1988). These data suggest a strong correlation between the expression of c-fos and ongoing neuronal activity, and indicate that Fos immunostaining may provide a powerful tool for mapping the pattern of postsynaptic neuronal activation with single cell resolution.

Prostaglandin E2

Biological properties of PGE2 actions

Prostaglandins, because of the widerspread distribution of their receptors, have been implicated in such physiological processes as nerve transmission, reproduction (Kennedy 1977, 1978, 1980), gastric secretion, vasoconstriction, bronchoconstriction (Horton 1972; Cuthbert 1974), platelet aggregation (Smith and Macfarlane 1974), inflammation, fever (Nakamur et al, 1988) and pain (Watkins et al, 1994). Synthesis of prostaglandins in cells and tissues is evoked by a plethora of membrane-perturbing stimuli, including IL-1 (e.g. by binding IL-1 receptors) and TNF alpha (Akama et al, 1990; Topley et al, 1989; Uotila and Vapaatalo, 1984; Betz and Fox, 1991), trauma and injury (Horgan, 1994), hemorrhage (Coimbra et al 1996), infection (Clerici et al, 1993), mitogens (e.g.concanavalin A) (Fraifeld et al, 1995), endotoxins (LPS), antigen/antibody complexes (Berger et al, 1996), the cross-linking of Fc receptors, components of the complement cascade, lead (Lee and Battles, 1994), histamine, neural, hormonal, etc. The almost ubiquitous occurrence of the prostaglandin synthetase enzyme system together with the presence of its substrate fatty acids in membrane phospholipids of mammalian cells, suggests that prostaglandin could be formed within all cell types (except erythrocytes). There is little evidence in the literature that prostaglandin accumulates intracellularly (Wolfe, 1978) with the exception of seminal vesicles, which are probably the only storage sites of prostaglandin in the body (Bergstrom 1974). Also, semen contains large amounts of prostaglandin (Hamberg and Samuelsson 1966) and urine contains prostaglandin and

prostaglandin metabolites (Frolich et al 1973). In adult human blood plasma basal levels of prostaglandin normally are in the subnanomolar range (Silver et al 1972).

Prostaglandins can be classified according to the number of double bonds in the side chains. Prostaglandin E2 (PGE2) contains 2 double bonds at positions 5, 6 and 13, 14. It has a keto group in the 9 position and an hydroxyl group in the 11 position. The rate-limiting step in the biosynthesis of PGE2 is the release of arachidonic acid from membrane-bound phospholipids. The agent responsible for this release is phospholipase A2, an enzyme activated by chemical, immunological or mechanical stimuli that disrupt the cell membrane. Once freed from membrane-stores, arachidonic acid is converted either to leukotrienes by lipoxygenase, or to prostaglandin endoperoxides by cyclooxygenase. Among the endoperoxides formed through the action of cyclooxygenase is PGG2, which is converted to PGE by PGE2 isomerase (Moore, 1985; Wolfe, 1982). The biological effects of PGE2 is couple with cyclic adenosine monophosphate (cAMP), which is an intracellular second messenger that is known to convey inhibitory signals for immune cell proliferation and function (Horgan et al, 1994).

PGE2 and the regulations of cytokines and lymphoctyes

PGE2 is a potent lipid molecule with complex pro-inflammatory and immunoregulatory properties. The production of PGE2 can be induced or increased by many factors as mention before such as cytokines etc, but there are many protective negative feedback loops of physiological systems in the body to prevent unlimited amplification in the production of PGE2. PGE2 can decrease TNF-alpha and other pro-

inflammatory cytokines in monocytes/macrophages (Kunkel et al, 1988; Taupin etal, 1992). However, IL-1 beta and TNF alpha can also stimulate IL-8 formation, a chemokine, and the IL-8 can stimulate LTB4 formation (Schroder 1989) which may competitively inhibit PGE2 formation by utilizing arachidonic acid. Although PGE2 can enhance IL-4 formation, IL-4 has a negative feedback effect on PGE2's formation. Because IL-13 and IL-4 share many biologic activities and structural and functional properties (Punnonen et al, 1993; McKenzie et al, 1993), such as a common signaling receptor subunit (Zurawski et al, 1993; Aversa et al, 1993), it is possible that PGE2 can stimulate IL-13 formation. There is evidence that IL-13 can have a negative feedback effect on PGE2 (Onoe, 1996). Furthermore, PGE2 can augment immune complex-induced IL-6 and IL-10 secretion, but IL-10 is capable of down-regulating the release of PGE2 and the secretion of proinflammatory cytokines (Berger, 1996).

CD4 T-helper cells (Th1 and Th2) play a pivotal role in the regulation of specific immune responses. PGE2 can modulate the cytokine secretion pattern from T-helper cell subpopulations via an increase in cAMP and shape the immune response by synthesis of Th2 cytokines [e.g., IL-4, IL-5, IL-10, IL-13], while inhibiting production of Th1 cytokines [e.g., IL-2 and IL-12 interferon-γ] (Betz and Fox, 1991; Paliogianni and Boumpas, 1996). Research reports have showed that PGE2 can enhance IL-4 and IL-5 production (Phipps, et al, 1991) at physiological concentrations (10^{-8} to 10^{-7} M) (Hilkins et al, 1996).

Additionally, Garrone and his colleagues (1994) have suggested that PGE2 at physiological concentrations can regulate the differentiation and proliferation of human B

lymphocytes activated through their CD40 antigen, but not their Ig secretion. Addition of PGE2 can further potentiate both IL-4- and IL-10-induced B cell growth, but IL-4-induced IgG and IgE secretion is inhibited, while the IL-10-induced IgM, IgG, and IgA secretion is greatly enhanced. It appears that in order for PGE2 to enhance Ig secretion and alter Ig isotypes, it requires other co-factors (e.g. cytokines). Finally, the effects of PGE2 are mimicked by agents that increase cAMP, indicating that many of the actions of PGE2 are likely to depend on the activation of the cAMP pathway. Altogether, in microenvironments supporting the development of an immune response, the secretion of PGE2 by competent cells such as macrophages may participate in the regulation of the cytokines and lymphocytes .

The role of PGE2 in neuro-immune regulation

PGE2 plays a crucial role within the CNS in the interface between the immune and nervous systems (Rassnick et al 1995). PGE2 mediates the stimulatory effects of cytokines on hypothalamic-pituitary-adrenal (HPA) function (Watanabe etal, 1990), the inhibitory effects of cytokines on the activity of the hypothalamic-pituitary-gonadal axis and on motivated behavior.

Experiments have shown that intracerebroventricularly (ICV) administration PGE2 produces a marked decrease in both blood lymphocyte responses to the T lymphocyte mitogens PHA, which predominantly stimulates CD4 T cell, and ConA, which predominantly stimulates CD8 T cell. Also central PGE2 injections produce a decrease in the proliferative responses to LPS, which stimulates proliferation of B cells (Rassnick

1995). The decrease in mitogenic responses of blood lymphocyte after ICV PGE2 also is associated with increases in plasma concentrations of ACTH and corticosterone (Watanable et al, 1990), which resemble some of the hormonal responses to stress. Also ICV PGE2 increases prolactin and growth hormone levels (Ojeda et al, 1980). We can exclude the possibility of PGE2 acting in the periphery, since only 0.07-0.13% of an administered IV dose enters the brain (Eguchi et al, 1988). When PGE2 is synthesized in vivo and enters the blood circulation (Cuthbert 1974), PGE2 is rapidly metabolized and does not remain in the circulation long enough to act as a classical hormone (Horton 1972; Vane 1969). These results provide the basis for the hypothesis that the CNS mechanisms of immune system regulation involve PGE2. The data are consistent with the idea that the decreased mitogenic responses of blood and spleen lymphocytes are mediated by increased activity of the sympathetic nervous system and the HPA axis (Cunnick et al 1990; Wan et al 1993; MacNeil et al 1997) and PGE2 functions as part of the signaling pathway for activating the neural-immune regulatory system.

Effects PGE2 on CRF and PAF

Recent evidences suggest that not only the end product of the hypothalamic-pituitary-adrenal axis, but also other hormones in the axis may be involved in regulation of the inflammatory response. Corticotrophin-releasing factor (CRF), a 41 amino acid peptide, was originally isolated from the hypothalamus and named because of its property to stimulate the anterior pituitary secretion of adrenocorticotrophic hormone. Both PGE2 and IL-1 can induce the expression of CRF (Petraglia et al., 1989,1992).

Recent data have shown that CRF acts as an autocrine/paracrine pro-inflammatory regulator in addition to its role as an endocrine hormone, and it can be produced by various cell types in several extrahypothalamic sites (Makrigiannakis et al., 1995b, Mastorakos et al., 1993, Petraglia et al., 1992, Fabbri et al., 1990, Grino et al., 1987, Frim et al., 1988). CRF appears to play an important role in the initiation and propagation of an inflammatory response and it can stimulate leukocytes to produce IL-1 and act in many respects as an inflammatory mediator (Karalis et al, 1991). CRF exhibits a strong vasoactivity in skin tests and systemic administration induces an increase in capillary permeability and vasodilation(Hermus et al., 1987). In hypothalamic explants, the secretion of CRF was found to be stimulated by IL-1, TNF alpha or IL-6. PGE2 as well as platelet-activating factor (PAF), act as amplifiers of this secretion (Navarra et al, 1991; Bernardini et al, 1990). Fleisher-Berkovich and Danon (1995) investigated the role of CRF in the regulation of PGE2 synthesis in fibroblasts and endothelial cells. They found that CRF suppresses IL-1α-induced PG synthesis through actions on both phospholipase A2 and cyclooxygenase.

Recent research reports have indicated the effect of central administration of the PGE2 on the transcriptional activity of corticotropin-releasing factor (CRF) and its type 1 receptor in the brain of conscious rats (Lacroix et al, 1996). Activation of CRF neuroendocrine cells is also associated with an increase in CRF transcription as revealed by the selective presence of CRF primary transcript (hnRNA), which was stimulated only in the PVN but not in any other nuclei in the brains of PGE2-treated rats. Central administration of PGE2 also induced expression of the CRF type 1 receptor in the

parvocellular PVN. Altogether, in terms of the essential roles of PGE2 induction of CRF/ACTH release, these findings suggest a novel regulatory cascade in immune-neuroendocrine interactions.

Platelet-activating factor (PAF) , in addition to leukotrienes and PGE, is one of the most important lipid mediators in inflammatory disorders. PAF was first described as originating from sensitized basophils challenged with a specific allergen but it is now known that PAF is generated de novo by macrophages (Arnoux et al. 1980), Neutrphils (Lotner et al.1980) and other inflammatory cells. PAF shows various biologic effects such as activation of inflammatory cells (Yasaka et al. 1982; Shaw et al. 1981), elevation of vascular permeability (Humphrey et al. 1984), induction of hypersecretion by submucosal glands (Hahn et al. 1985) and platelet aggregation. PAF is considered to be one of the most potent inflammatory mediators. Other data have indicated a paracrine interaction of PAF. PGE2 can be released by hormonally sensitized cells under the influence of PAF (Thivierge and Rola-Pleszczynski 1995). In turn, PGE2 can induce PAF production (Kasamo et al.1992; Van der Weiden et al.1991; Harper 1989; Alecozay et al.1991, 1989). In inflammatory site, both PGE2 and PAF can be detected (Jung TTK 1988) .

Central effect of PGE2 on fever

As described before, PGE2 like IL-1 and TNF, has many central effects (Sapolsky, et al. 1987; Besedovsky, et al., 1986; Kabiersch, et al., 1988; Zalcman, et al., 1994). For example aspirin-like drugs and indomethacin have antipyretic effect and act by blocking

the cyclooxygenase activity involved in the production of prostaglandin (Avery and Penn 1974).

Fever is produced by the coordinated actions of many CNS regions as an adaptive response to infection. This essential role for prostaglandin in fever was proposed 26 years ago to explain the observation that systemic inhibition of prostaglandin production blocked fever (Vane 1971). Thus PGE2 is a pyrogen-like mediator. As the autocoid elevates the body temperature set-point to evoke the autonomic responses characteristic of fever, a cascade of events occur. Beginning with endotoxin (foreign pyrogene) and proceeding to the synthesis and release of pro-inflammatory cytokines (TNF-belta, IL-1 alpha and IL-6), the cytokines are thought to stimulate PGE2 production within the organum vasculosum laminae terminals region. PGE2 then may act as a local febrigenic mediator or act in the surrounding preoptic area (POA) and diffuse across the blood-brain barrier into the MPOA and the organum vasculosum of the lamina terminalis (OVLT) (Dascombe and Milton 1979; Morimoto et al 1987), and then activate neurons in the ventromedial preptic (VMPO), which, in turn, stimulates the paraventricular nucleus of the hypothalamus (PVH) (Scammell et al 1996). This upward displacement of the set-point produces fever (Stitt 1986; Arend et al 1989; Ashman and Mullbacher 1984; Ater et al 1985; Skarnes et al. 1981). Recent reports have suggested that some peripheral immune cell-derived signals may be transmitted via the vagi to the medulla. This information may be conveyed via the central noradrenergic bundle to the POA region, where the released of norepinephrine induces the local synthesis of PGE2 and the onset of fever (Sehic and Blatteis 1996). Additionally, many autonomic regulatory structures can be activated

during PGE2 induced fever in a pattern similar to that seen during the fever produced by systemic administration of LPS (Elmquist et al 1996). In summary of the effect of PGE2 on fever, we can assume that PGE2 in cooperation with the cytokines induced by LPS, transmits signals outside the blood-brain barrier to neuronal circuits, thereby mediating communication between the immune system and the CNS.

Effects of PGE2 on diseases

Many inflammatory diseases begin with fever. Fever is closely associated with host-defense mechanisms, an important function of immune system. PGE2 can have an amplifying effect on fever induced by pro-inflammatory cytokines. Retrospective clinical studies have suggested that patients who had a moderate fever (100-101 °F) survived bacterial peritonitis (Hoefs et al. 1980) and bactermia (Bryant et al. 1971) in greater numbers than those who failed to develop fever. Kurosawa and his colleagues (1987) infected rabbits with rinderpest virus, and examined the outcome of the disease in those treated with an antipyretic (either mefanamic acid or acetylsalicylic acid) and those left untreated. The use of antipyretic and the attendant reduction of fever not only increased the number of animals succumbing to infection, but retarded recovery among those that did survive (For review see Ref. Hart 1988)

From recent data in the literature, it is possible that for many autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus, the pyrogenic role of PGE2 has an amplifying function on the immune system, and may further enhance the activity of an abnormally active immune system (Duff 1986). At a moderate febrile temperature of 39 °C, the IL-1 driven T cell proliferative response can be as much as ten-fold greater

relative to a normothermic incubation temperature, and the effects are mainly on T-helper type 2 cells (Duff and Durum 1982, 1983; Jampel et al. 1983). PGE2 can always be located at inflammatory sites. Studies have shown that significantly more PGE2 can be found in the patients with human immunodeficiency virus infection (Clerici et al. 1993), IgE-type allergic diseases (Jakob et al. 1990, Chan et al. 1993), rheumatoid arthritis of autoimmune disease (Duff 1986) and cancer (Uotila 1993; Arvind et al. 1995; Huang et al. 1996; Handel-Fernandez et al. 1997), relative to control subjects. Because PGE2 favors TH2-like cytokine secretion profiles by inhibiting the production of the Th1-associated cytokines (Betz and Fox 1991, Paliogianni and Boumpas 1996), this may explain why PGE2 may worsen some diseases. In another example of IgE-type allergic diseases, Takenaka (1995) reported that cross-linking of the Fc epsilon R I and IgE can induce PGE2 release from monocytes of atopic dermatitis. In addition, there was a trend towards an age-associated increase in PGE2 production in response to mitogenic stimuli. (Fraifeld et al. 1995). However, Snyderman et al (1995) recently showed that although PGE2 may have adverse affects on local immune function in tumor tissues, improved survival of patients with increased local PGE2 production may be indicative of an enhanced immunologic response to the tumor which has a favorable impact on outcome.

Actions of PGE2 at the molecular level

1. Expression of oncogene in the brain:

Lacroix and his colleagues(1996) have investigated, using immunocyto-chemistry and in situ hybridization techniques, the effect of central administration of the PGE2 on

the distribution of the immediate early gene (IEG) c-fos mRNA in the brain of conscious rats. In their experiments, thirty min after PGE2 injection, a moderate to strong positive signal for c-fos mRNA was detected in multiple structures of the brain such as the medial preoptic area/organum vasculosum of the lamina terminalis, supraoptic nucleus (SON), parvocellular and magnocellular divisions of the paraventricular nucleus (PVN) of the hypothalamus, central nucleus of the amygdala, nucleus of the solitary tract, dorsal motor nucleus of the vagus, area postrema, dorsal division of the ambiguus nucleus, and throughout the choroid plexus and leptomeninges. A smaller but significant c-fos expression was observed in other structures including the subfornical organ, bed nucleus of the stria terminalis, arcuate nucleus, and periventricular nucleus of the hypothalamus. Two hours after treatment with the PGE2, the signal for c-fos mRNA in most of these brain nuclei vanished. In the parvocellular nucleus of the PVN, c-fos mRNA was expressed in CRF-immunoreactive (ir) and OT-ir neurons, whereas in the magnocellular part of that nucleus and in the SON, this transcript was essentially colocalized in OT-ir neurons. This result is consistent with our early experiments in which pretreatment with the PGE2 synthesis inhibitor, indomethacin, blocked the activation of c-foc protein in the hypothalamus produced by both IP or IV injections of LPS (Wan, et al., 1994).

Brown and Phipps (1996) examined the differential sensitivity of immature B cell lymphomas to growth inhibition by PGE2. They found that PGE2 can increase the percentage of cells in G1, and analysis of synchronized populations revealed that PGE2 acts at all phases of the cell cycle to delay normal progression. Apoptosis induced through PGE2 and cAMP signaling is sensitive to regulation by Bcl-2 in the immature B cell lymphomas. Bcl-2 can inhibit PGE2- and cAMP-mediated DNA fragmentation and nuclear condensation.

2. Trans-action elements:

PGE2 can modulate the activity of a number of transcription factors and inhibit the nuclear transcription of IL-2 gene in primary human T cells. The IL-2 promoter contains binding sites for nuclear proteins such as Activating Protein-1 (AP-1), Nuclear Factor of Activated T cells (NF-AT), Octamer-1 (Oct-1). Felli and his colleagues (1996) recently have suggested that the octamer-octamer interaction is a novel target of the PGE2-induced down-regulation of the IL-2 promoter. In their experiments, PGE2 can inhibit T cell activation and the transcription of the IL-2 gene through down-regulation of the octamer-dependent transcription of the octamer motif. Thus, by interfering with signals activating the octamer motif, this motif can bind Oct-1 and Oct-2 as well as the phorbol ester and calcium ionophore-inducible jun and fos activating protein-1 (AP-1) factors. The PGE2-dependent down-modulation was observed in the presence of either the endogenous transacting factor Oct-1 or the exogenously expressed Oct-2. Furthermore, Paliogianni and Boumpas (1996) also found that cAMP regulates IL-2 production in human T cells by a transcriptional mechanism which involves discrete transactivating pathways for IL-2-promoter activation. Using DNA transfection and electrophoretic mobility shift assays (EMSAs), nuclear extracts from primary human T cells stimulated with ionomycin and phorbol esters in the presence of PGE2 demonstrated decreased binding at the AP-1 and NF-AT sites of the human IL-2 promoter binding to the OCT-1. They presented evidence that PGE2 can inhibit the nuclear transcription of the human IL-2, but not the Il-4 gene, by targeting transcription factors AP-1 and NF-AT.

PGE2 is pleiotropic and exerts stimulatory as well as inhibitory actions on immune responses. Micali et al (1996) have demonstrated that a short treatment of human T lymphocytes with PGE2 induces specific binding activity to cAMP-regulated enhancer (CRE) and AP-2, but not AP-1, DNA elements. Okadaic acid, a potent protein

phosphatase inhibitor, prolongs the induction of the binding activity during which phosphorylation events are likely to occur. This activity is due to increased cAMP levels in that forskolin and IBMX mimic the effects of PGE2. Finally, by using transfection experiments with CRE-CAT plasmid, they found that PGE2 can activate the transcription of a CRE-containing promoter.

THE PRESENT STUDY

We have shown that sympathetic nerves innervate the spleen in the rat via the splenic nerve (Nance and Burns 1989), stress can affect splenic immune function and induce c-fos expression in the rat brain, central and peripheral administration of endotoxin induces c-fos immunoreactivity in hypothalamus and brain stem nuclei (Wan et al 1992, 1993, 1994), bacterial endotoxin can activate splenic sympathetic nerve electrical activity and this increased splenic sympathetic nerve activity was shown to be mediated by central prostaglandin synthesis (MacNeil 1996, 1997). The present study is a direct extension of our research. I have examined the effects of central administration of PGE2 prior to a peripheral injection of bacterial endotoxin on plasma corticosterone levels, c-fos protein expression in the hypothalamus, and cytokine mRNA levels in the spleen. In addition, I have tested the effects of cutting the splenic nerve on endotoxin induced splenic cytokines levels. The hypothesis is that PGE2 acts as a central mediator which activates central neurons that activate the HPA axis and increases sympathetic nervous activity. Support for the hypotheses would be that splenic cytokine levels are suppressed by central injection of PGE2 and cutting the splenic nerve would abrogate this suppression.

In the first experiment, animals were injected ICV with 4μg/20 μl of PGE2 or saline. At 30 minutes post injection, all animals were injected IV via the tail vein with 0.1μg of LPS. At 1 hour post injection of LPS, animals were overdosed with sodium pentobarbital and spleens collected for TNF-alpha and IL-1 beta mRNA levels as an index of immune cell activation, and blood samples (via cardiac puncture) collected for plasma corticosterone analysis. It was predicted that PGE2 would induce c-fos protein in the

hypothalamus,decrease splenic immune cell function and elevate plasma corticosterone. In the second experiment, the rats were divided into two groups composed of sham surgeries or splenic nerve sections. Rats were given an ICV injection of the PGE2 (4 μg in 20 μl saline) and 30 minutes later injected iv with 0.1 μg LPS. It was predicted that the immune effects of central PGE2 on splenic immune function was mediated by the splenic sympathetic nerve.

MATERIALS AND METHODS

Experimental Animal

The animals used for these experiments were 40 experimentally naive, male Sprague-Dawley rats, obtained from Charles River, Dowal Quebec, and weighing 250-270 g. The rats were housed individually in polypropylene cages and maintained on a 12:12 hour lighting regime. Tap water and Purina Rat Chow were provided ad libitum. Animals were routinely handled so that baseline corticosterone values would remain low during each experiment.

Anaesthetic

All surgical procedures were carried out under Somnotol (Sodium pentobarbitol, 60mg/kg, MTC Pharmaceutical, Cambridge, Ont.) anesthesia. The anesthetic was administrated intraperitoneally using a sterile tuberculin syringe.

Surgery

Intraventrucaler cannulation.

Seven days prior to each experiment, all animals were anesthetized and placed in a Trent Wells stereotaxic apparatus with the incisor bar placed 2.4 mm above the interaural line (Paxinos and Watson 1986). An incision of approximately 2.5 cm in length was made

and the skin retracted to expose connective tissue. The skull was scraped to remove the connective tissue. Bregma was marked on the skull and a small hole in the skull was made with a dental drill. A cannula was stereotaxically implanted in the lateral ventricle of each rat with coordinate 1.0 mm posterior to bregma, 1.4 mm lateral and 4.9-5.5 mm deep. Cannulae were fixed to the skull using stainless steel screws and dental cement. Animals were food restricted 12 hours prior to surgery and prior to anesthesia were injected SC with 0.02 ml atropine (0.5 mg/ml) to reduce respiratory problems during anesthesia. Immediately after surgery, they were injected IM with a spectrum antibiotic (0.04 ml, penicillin).

Splenic denevation.

The rats (n=20) which had been implanted with cannulae in the lateral ventricle were divided into two groups: sham operated and splenic denervation. They were deeply anesthetized with Somnotol and a large incision was made in the left posterior lateral abdominal wall. The slpeen was retracted and by means of blunt dissection under a dissecting microscope, the splenic artery and its multiple branches were exposed. The splenic artery was separated from the splenic vein and the surrounding connective tissue. Generally two nerves could then be visualized running on the surface of the artery prior to the point where the splenic artery divides into multiple branches which continue on to the spleen. All nerves branches were cut with microscissors at two points and a 5 mm segment of nerve removed. Sham operations were similar except that the nerves were not cut. The muscles and skin were then apposed and sutured.

Procedure

Injection:

In the first experiment, the rats (n=20)which were not been splenic denevation were divided into two groups. One groups were infused ICV with 4μg/20 μl of a prostanglandin E2 type (long-active, 15-methy-PGE2, Caynon) in 20 μl saline and the other group infused ICV with 20 μl saline. At 30 minites post injection, all animals were injected IV via tail vein with 0.1μg/0.02ml of LPS (E. coli serotype 055:B5 Sigma St Louis,MO).

In the second experiment, the rats(n=20) were divided into two groups composed of sham surgeries and splenic nerve sections. All rats were given a ICV injection of PGE2 (4 μg in 20 μl saline) and 30 min later they were injected iv with 0.1 LPS in 0.02 ml saline.

Histological Procedure:

At 1 hour post injection of i.v. LPS or saline, animals were overdosed with sodium pentobarbital and spleens and blood (via cardiac puncture) collected. The portion of the spleen closest to the splenic artery was removed and immediately frozen in liquid nitrogen for Northern Blot analysis of TNFα mRNA and IL-1β mRNA. The blood was collected in microtube containing 20 μl 0.5 EDTA (pH 8.0). The blood was centrifuged at 2000 RPM for 15 min and the supernatant removed and frozen for the corticoterone assay. Animals were perfused transcardially using 120 ml of 1.0% sodium nitrite followed by 300-500 ml of 4.0% fresh paraformaldehyde in 0.1M Phosphate buffer (pH=7.2) after collecting the spleen and the blood. Brains were removed, post-fixed for 3-4 hours and then cryoprotected in 30% sucrose in 0.1M phosphate buffer (pH 7.2) for 48 hours.

Frozen sections of brain were cut at 40 μm coronally on a freezing microtome, collected into 24 well culture plates containing 0.01M phosphate buffer saline (PBS) and rinsed for 30 min on an aliquot rocker (Miles). The alternate sections were utilized for immunocytochemical procedures.

Immunocytochemical Procedure

Frozen sections were collected and rinsed in 0.01 M PBS three times (3x10min), tissue sections were incubated in an affinity purified polyclonal rabbit anti-c-fos antibody (Santa Cruz Biotechnology, Inc) which was diluted 1: 7500 in 0.01M PBS + 1.0% Triton x100 (T-octylphenoxypoly-ethoxyethanol) + 1.0% NGS (normal goat serum, Cappel Research products, Scarborough, Ont.) + 2% BSA (Albumin, Bovine. Sigma Chemical Co.). Sections were gently agitated on a aliquot rocker overnight (12-18 hours) at room temperature. The following day sections were rinsed three times (3x10 min) in 0.01 PBS, incubated in GAR (goat anti-rabbit IgG, Cappel, Scarborough, Ont.) diluted 1:150 in 0.01M PBS 1% Triton x 100 + 1.0% NGS for 90 min. Sections were again rinsed three times in 0.01 M PBS and incubated for 90 min in rabbit PAP (Cappel, Scarborough, Ont.) diluted 1:300 in 0.01 M PBS + 1% Triton x 100 + 1.0 NGS. Sections were then washed (3x10min) in 0.01 M PBS and transfered to plates containing the chromogen diaminobenzidine, d-glucose for 5 min, after which glucose oxidase (Sigma, St. Louis, MO) was added to the solution. The peroxidase reaction was visually monitored for 30-40 min at room temperature. Sections were rinsed three times (3X10 min) in 0.01 M PBS, mounted onto subbed slides, air dried, cleared in alcohol/xylene, and coverslipped with Permount (Fisher Sci).

Brain sections taken at comparable levels of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus of each animal were used to index the number of c-fos-labelled cells for each group. The most heavily labelled single-section through the PVN or SON of each animal was selected for cell counts. All cells labelled in a single brain section were counted, regardless of the intensity of staining, in a standardized manner through a microscope using a grid reticule analyed by one way ANOVA.

Northern blotting:

Total RNA was isolated from fresh frozen rat spleens with TrizolTM reagent (Life Technologies, Burlington, ON) according to the manufacturer's instructions. The RNA samples were then denatured (50% formamide/MOPS/17.5% formaldehyde/10% Glycerol and Ethidium bromide) for 10 minutes at 65 C and run on a 1.2% agarose/formaldehyde gel for 3 hours at 50V.The RNA was transferred overnight to nylon membrane (Boehringer Mannheim, Laval, PQ) by capillary action with SSC buffers. The membrane was baked for 2 hours at 80 degrees to immobilize the RNA, afer which the ethidium bromide-stained 28s and 18s ribosomal RNA bands were visualized using a UV transilluminator to verify the integrity of samples. The membranes were equilibrated with 2x SSC and prehybridized for 1 hour at 42 degrees (in 50% formamide, 5x SSC, 0.1% sodium laurosarcosine, 0.2% SDS, and 2% Boehringer block). The membranes were then hybridized with biotin-or Digoxenin (Dig)-labelled riboprobes overnight at 60 degrees. After hybridization, membranes were washed twice in 2x SSC/0.1% SDS for 15 min at room temperature and twice in 0.1 x SSC/ 0.1% SDS at 60 degrees for 25 minutes. Membranes were blocked for 1 hour in 1% Boehringer block dissolved in maleic acid buffer, incubated with sheep anti-Dig peroxidase-conjugated antibody (1/5000, Boehringer

Mannheim). Following three rinses in maleic acid buffer, the Dig-labelled bands were visualized with a chemiluminescent substrate for peroxidase (Boehringer Mannheim).Chemiluminescent signals were exposed to film anywhere from 15 seconds to 30 minutes. Solutions were treated with 0.1% DEPC as requied. After chemiluminescent detection, the membranes were again prehybridized for one hour and hybridized with DNA probe randon-primed with ^{32}P DCTP (NEN) for ribosomal RNA or GAPDH.

For statistical analysis data values are expressed as means $\pm$standard error of the mean and a one way ANOVAs.will be performed (by group) on the densitometry data generated by Northern Blots.

Corticosterone Radioimmunoassay

Vials of rat serum and standards were thawed and 5 µl added to 0.5 ml assay buffer (in distilled water, 0.82% anhydrous sodium acetate, 0.01 % sodium azide), and pH adjusted to 5.2 with acetic acid. After pH adjustment 0.1% bovine serum albumin (Sigma chemical Co.)was added. Duplicate 100 µl volumes of unknown and standard dilutions were placed in glass tubes and 100 µl of rabbit anti-corticosterone antiserum (Sigma) was added (diluted 1:5 with physiological saline containing 0.1% sodium azide and then further diluted 1:60 with assay buffer). Following gentle mixing, 100 µl of 3H corticosterone (New England Nuclear, No. NET399; 25 uCi in 7.5 ml re-distilled ETOH then diluted with assay buffer so that 100 µl yielded approximately 25,000 cpm) was added to all tubes, mixed, and incubated at 4 ^{o} C for 90 min. Following incubation, 1 ml of charcoal/dextran (0.025% dextran T-70 and 0.25% Norit A charcoal in assay buffer) was added to all tubes, mixed well, incubated at 4 ^{o} C for 10 min, and then centrifuged at 3000 rpm for 15 min in at 4 ^{o}C centrifuge. Supernatants were decanted into scintillation vials and 5 ml Universol (ICN Biomedicals, Irvine, CA) added. Vials were mixed vigorously for

30 min, allowed to stand for overnight in the dark at room temperature and then counted in a Beckman ß scintillation counter for 10 min or 104 counts. Corticosterone levels were statistically compared by a one way ANOVAs.

RESULTS

ICV PGE2, saline and IV LPS experiment

Distribution of labeled c-fos positive cells:

Brain sections were examined with light microscopy and representative sections were selected for microphotography.

The patterns of c-fos immunostaining in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus were comparable to that decribed previously for LPS and stress (Wan, W. et al. 1993). Relative to vehicle-injected controls, there was a dramatic increase in the number of c-fos-positive neurons localized in the PVN and SON following ICV injections of PGE2 and IV injections of LPS (Fig1).

Inserted Fig 1 here

As illustrated in Fig 2, ICV injections of PGE2 produced significantly more c-fos-labeled neurons in the PVN than did saline ICV injections, $F(1,18)= 78.88$, $p< 0.001$.
The Fig 3 also demonstrates that ICV injections of PGE2 produced significantly more c-fos-labeled neurons in the SON than did saline ICV injections, $F(1, 18)=236.485$, $P<0.0001$.

Inserted Fig 2, Fig 3 here

Corticosterone Production:

There was a small difference in plasma corticosterone production with PGE2 injected animals showing a higher level of corticosterone, relative to the saline group; but, this difference was not significant, $F(1, 18) = 2.9043$, $p = 0.1282$.

Fig 4 Effect of the ICV injections of PGE2 on plasma corticosterone levels.

Inserted Fig 4 here

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splenic cytokine production:

Central injections of PGE2 produced a dramatic supression in the in vivo induction of TNF-α mRNA in the spleen of rats following an IV injection of endotoxin, $F(1,18) = 4.0042$, $p < 0.05$.

Fig 5 illustutes the effect of the ICV injections of PGE2 on TNF-α mRNA levels relative to saline group, 30 min after ICV injections and one hour after IV LPS. (*) indicates PGE2 group is statistically significant from the saline control.

Inserted Fig 5 here

Fig 6 This graph demonstrates the effect of the ICV injections of PGE2 on IL-1beta mRNA levels, relative to saline group, 30 min after ICV injections and one hour after IV

LPS. This was no difference between the PGE2 and saline groups, F(1,18)=1.2396 p=0.8488.

Inserted Fig 6 here

Splenic nerve cut, ICV PGE2 and IV LPS experiment.

Distribution of labeled c-fos positive cells:

Fig 7 Comparison of c-fos-labeled neurons in the PVN from sham and splenic nerve cut after ICV injections of PGE2 and IV LPS. The two groups did not have difference in terms of the number of c-fos neurons expressed, F(1,11)=2.243 P=0.3536.

Fig 8 shows the comparison of c-fos-labeled neurons in the SON from sham and splenic nerve cut the two groups didn't have difference from each other, F(1,11)=3.0318 P =0.21.

Inserted Fig 7 and Fig 8 here

Corticosterone Production:

Fig 9 Comparison of plasma corticosterone levels from sham and splenic nerve cut rats after ICV injections of PGE2 and IV LPS. Their was no statistical difference between splenic nerve cut and sham operated groups, F(1,18)=1.3701 P=0.6638

Inserted Fig 9 here

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splenic cytokine production:

As illstrated in Fig 10, the suppressive effect of the ICV injections of PGE2 on IV LPS induced TNF-αwas attenuated in the nerve cut group which showed significantly higher splenic TNF-α mRNA levels than the sham operated group. $F(1,18)=11.9162$ $p<0.01$

Inserted Fig 10 here

Fig 11. This graph demonstrates the effect of the ICV injections of PGE2 on IL-1 β mRNA levels between splenic cut and sham groups 30 min after ICV injections and one hour after IV LPS. The groups were not statistically different, $F(1,18)=1.0534$ $p=0.9326$.

Inserted Fig 11 here

DISCUSSION

There are two functional pathways from the brain to the periphery that are known to inhibit immune function. One pathway consist of the activation of the hypothalamic-pituitary-adrenal (HPA) axis which produces an increase in plasma corticosterone, a known immunosuppressive steroid (Bateman et al 1989). The other pathway is the sympathetic nervous system (SNS), which increases sympathetic outflow and may increase NE synthesis, turnover and release in the spleen. NE is also an immunosuppressive mediator (Madden and Livnat 1991). The data in this study provides strong evidence that centrally injected PGE2, followed by IV LPS, decreases TNF-alpha expression in the spleen and cutting the splenic nerve abrogates this suppression.

Centrally administered PGE2 activates the HPA axis:

The hypothalamus is a central part of brain in that it integrates endocrine, autonomic and behavioral response by direct control of the pituitary gland and by direct modulation of preganglionic autonomic neurons in the brain stem and spinal cord (Swanson 1985). The neurosecretory cells controlling the HPA axis are located in the PVN of the hypothalamus. Following iv LPS, we investigated the role of icv PGE2 in inducing c-fos expression in the PVN and SON of the hypothalamus. We found that these regions of the hypothalamus showed a robust c-fos expression after ICV PGE2. Many neurosecretory cells in PVN are CRF neurons and both LPS and IL-1 induce an

activation of CRF cells in the PVN (Ericsson et al 1994; Rivest and Laflamme 1995). Interestingly, irPGE2 immunoreactiviting following LPS treatment is limited mainly to the magnocellular cells of PVN and SON (Van Dam et al 1993). We can not exclude this possibility that the irPGE2 staining cells may belong to CRF neurons, because PGE2 can trigger the transcription of CRF and its receptor within the PVN and SON of the brain (Lacroix et al 1996). PGE2-induced release of CRF within these neuroendocrine regions may play an important part in mediating interactions between the brain and the immune system.

The production of c-fos protein in CRF neurons results in the initiation of CRF biosynthesis, and CRF further activates c-fos, CRF1 receptor transcription and CRF gene expression within the PVN of the hypothalamus (Mansi et al 1996; Parkes et al 1993). This positive feedback of CRF on its own induction in the PVN may represent a functional adaptation of the HPA axis in response to specific stimuli.

We previously demonstrated that c-fos protein was induced in the PVN and SON, as well as the A1 and A2 regions of the brain stem, by systemic injections of LPS (Wan et al 1993, 1994). Although the induction of c-fos in the brain produced by foot shock was unaltered by the cycloxygenase inhibitor, indomethacin, the indomethacin blocked c-fos expression in both the PVN and SON of the rat brain following LPS injections (Wan et al 1994). Our present results are consistent with these earlier data and show that PGE2 is a pivotal modulator in activating the HPA axis. Among the numerous intra- and extracellular mechanisms involved in regulating the HPA axis, PGE2 is a key mediator of various cytokines on the activity of neuroendocrine CRF neurons. For example,

blockage of the eicosanoid cyclooxygenase pathways can prevent the stimulation of CRF release by both IL-1 and IL-6 from in vitro hypothalamic explants (Navarra et al 1991; Lyson and McCann 1992) and median eminence (McCoy et al 1994), as well as IL-1-induced ACTH release in vivo (Katsuura et al 1988; Rivier 1993).

The PGE2 antagonist indomethacin infused into the organum vasculosum of the lamina terminalis and preoptic area (OVLT/MPOA) can significantly prevent the ACTH release following i.v. IL-1 beta administration in rats, and microinjection of PGE2 into the OVLT/MPOA increases plasma ACTH release (Katsuura et al 1990). Moreover, intracerebroventricular (i.c.v.) administration of PGE2 elevated plasma ACTH and corticosterone in rats (Rassinick et al 1995). These effects are most likely mediated through CRF neurons. Therefore, PGE2 could be a determinant mediator within the brain for stimulating the HPA axis during an immune challenge. It is clear that the PVN receives afferent innervation from the OVLT/MPOA (Sawchenko and Swanson 1983) and icv treatment with PGE2 causes transcriptional activation of neuroendocrine CRF neurons and stimulates ACTH and corticosterone release (Rassnick et al 1995). As previously reported in animals injected systemically with IL-1 (Ericsson et al 1994) or LPS (Rivest and Laflamme 1995), central treatment with PGE2 also caused expression of c-fos protein within the PVN and SON (fig.2, fig 3). The interaction between ascending NA fibers and PGE2 in mediating the activation of neuroendocrine CRF neurons could be located directly within the PVN. For example, injection of 6-OHDA into the PVN depleted NA content by 85% and reduced by 80-82% the increase in plasma corticosterone levels following i.p. injection of IL-1 (Chuluyan et al 1992). In addition,

the PVN receives a dense noradrenergic innervation from the A1 group of the caudal ventrolateral medulla. Neuropeptide Y (NPY) usually is colocalized with NA in the cells of the A1 group. Studies have showed that NPY triggers a release of CRF and elicits an increase in ACTH (Gartside et al 1995; Gaillet et al 1991; Tsagarakis et al 1989; Wahlstedt et al 1987). Together, these evidences suggest that PGE2 may act at the interface between circulating pro-inflammatory cytokines and neurons of the OVLT/MPOA involved in providing the information to the PVN to trigger the HPA axis.

Additionally, the c-fos expressing cells may be in oxytocin-immune reactive neurons of the magnocellular division of the PVN and the SON (Lacoix et al 1996). Apart from oxytocin (OT) being a family of neuropeptides able to potentiate the action of neuroendocrine CRF on the secretion of ACTH from corticotroph cells of the adenohypophysis during various types of challenges, OT can be secreted into the infundibular process and therefore be another candidate to help stimulating the HPA axis during an immune challenge.

Central injection of PGE2 increases splenic sympathetic nerve activity

Both pro-inflammatory cytokines and LPS induce a variety of host-defence responses including immunological, metabolic and cardiovascular modulations, which are not induced by simple thermal loads (Blatteis et al 1988; Dinarello et al 1988; Kulger et al 1991; Morimoto et al 1992; Sundar et al 1990; Take et al 1995). The involvement of the sympathetic nervous system (Brown et al 1991; Saito et al 1991; Take et al 1995), such as a sympathetic nerve-mediated downregulation of natural killer cell activity in the

spleen (Katafuchi et al 1993; Take et al 1995), might interact additionally in a regionally specific manner with sympathetic control as shown for renal, adrenal and splenic innervation (Niijima et al 1991).

The specific neuroanatomic pathways and neuro-tranmitters involved in sympathetic-immune interactions have been the focus of considerable research efforts. The general pathway of sympathetic outflow includes the PVN, the A5 cell group in the rostral ventrolateral medulla, and the ventromedial medulla and caudal raphe nucleus, all of which project to the sypathetic preganglionic neurons in the spinal cord (Sawchenko and Swanson 1982). Felten and his collegues (1987) verified noradrenergic sympathetic innervation of the spleen. Our laboratory proposed further that the spleen is exclusively innervated by sympathetic nerve fibers. Results from recent experiments indicated that sympathetic outflow can be directed to an immune organ in response to a stimulus known to activate the immune system (MacNeil et al 1996, 1997, Katafuchi et al 1993; Wan et al 1992; Vriend et al 1993). Specific doses of LPS can elicit specific responses from the SNS as recorded from the splenic nerve, whereas the stimulatory influence of IL-1 beta on SNS activity can be prevented by cyclooxygenase inhibitors (Ichijo et al 1994). Intracerebroventricular injection of PGE2, which mimics many of the effects of LPS and IL-1 (Ichijo et al 1994; Katafuchi et al 1993), resulted in a strong increase the activity of the splenic nerve (Ando et al 1995, MaNeil et al 1997). Several studies have documented the functional significance of splenic sympathetic innervation. The ability of stress to inhibit the lymphocyte proliferative response of splenic immune cells was prevented by cutting the splenic nerve prior to footshock exposure (Wan 1993). Central

injection of IL-1 beta increased splenic sympathetic nerve activity (SNA) (Ichijo et al 1994) and suppressed splenic NK cytotoxicity, and the immunosuppression was partially blocked by pretreatment with a sympathetic ganglion blocker (Sundar et al 1990). Also, the suppression of splenic immune responses (IL-1 and IL-2 production, natural killer activity, and lymphoproliferation) after central injection of IL-1 or interferon-gama was blocked by splenic nerve section or pharmacological blockade of synpathetic activity (Brown et al 1991; Sundar et al 1990;Take et al 1993). Moreover, electrical stimulation of the splenic nerve has been shown to reduce natural killer cell activity of splenic lymphocytes (Katafuchi et al 1993). Immune responses can be enhanced by surgical or chemical sympathectomy (Besedovsky etal 1979; Felten etal 1987). This observation support earlier findings that splenic sympathectomy can ablate immunosuppression (Brown et al 1991; Sundar et al 1990) and splenic deafferentation can block the stress-induced suppression of the plaque-forming cell response and T cell mitogen response of splenocytes (Wan et al 1992). It also confirms further that central PGE2 can stimulate splenic sympathetic nerve activity (Ando 1995) and the modulation of the splenic sympathetic activity may be brought about by a centrally induced autonomic outflow. Our results are consistent with these reports and provide strong support for our present results that central injection of PGE2 or i.v. LPS increases spleen nerve activity. In addition, in the parvocellular PVN, CRF-ir and OT-ir neurons expressed c-fos mRNA, whereas in the magnocellular PVN and the SON, the c-fos was primarily located within the OT-ir perikarya (Lacroix et al 1996). Thus PGE2 of central origin may be involved in the activation OT neurons projecting to the spinal cord to stimulate the SNS in immune-

challenged animals, and some of these OT-ir neurons project to dorsal vagal complex and the intermedio-lateral column of the spinal cord participate in regulating the sympathetic nervous system activity.

Central regulation of splenic TNF-alpha mRNA

It has been shown that i.v. IL-1 beta induces a signifincant increase in PGE2 levels both in the organum vasculosum laminae termminalis (OVLT), in which the blood-brain barrier is limited, and in the medial preoptic area (MPO), which is located close to the OVLT (Komaki et al 1992). Moreover, PGE2 induced by IL-1 beta in the hypothalamus stimulates secretion of corticotropin releasing factor (CRF) (Watanabe et al 1990) and CRF in the MPO stimulates sympathetic activity (Egawa et al 1990). In contrast to IL-1, it has been pointed out that the sympathetic modulation induced by TNF-alpha is mediated, at least partly, by a mechanism not related to the CRF pathway (Rothwell 1988).

In an acute inflammatory reaction, pro-inflammatory cytokines are usually secreted in a sequence, first TNF alpha, second IL-1 and then IL-6. Our result demonstrate that in the spleen, the regulation of TNF-alpha mRNA, but not IL-1 mRNA, is regulated by central PGE2 and the effects of cutting the splenic nerve verify further the differential neural regulation of these cytokines. Studies have found that either peripheral or central pretreatment with indomethacin attenuates, but does not inhibit completely, IL-1 induced fever (Hashimoto 1988, 1991). However, TNF-alpha induced fever is blocked totally by s.c. indomethacin alone (Watanabe 1992). Sympathetic nerve fibers innervate the spleen

and immunocompetent cells experess functional receptors for norepinephrine (Felten 1987). Thus, inhibiting immune function of spleen cells via the SNS is an important regulatory circuit from brain to periphery. In support of this, splenic norepinephrine levels decline preceding the proliferative phase of an immune response to sheep red blood cells (Besedovsky et al 1979). The pharmacological effects of beta-adrenergic receptors are coupled with adenylyl cyclase through G proteins which act as links in signalling across the membrance and produce cAMP. cAMP activates protein kinase A which leads to subsequent phosphorylation reactions and triggers calcium influx (Renter 1985). Interestingly, the actions of PGE2 is coupled with cAMP, which is an intracellular second messenger that is known to convey inhibitory signals for lymphocytes proliferation and function (Zhong et al. 1994; Horgan 1994) and for inflammatory cells function such as macrophages, basophils and neutrophils. There is increasing evidence that beta-agonists, through elevated cMAP levels, have anti-inflammatory and immunomodulatory actions. The intracellular concentration of cAMP is also regulated by phosphodiesterases (PDEs), a family of enzymes degrading cAMP. PDE inhibitors, such as theophylline, which elevate intraellular cAMP levels by PDE inhibition, have been found to inhibit TNF-alpha production by LPS-stimulated human monocytes. Furthermore, theophylline inhibited the production of TNF-alpha by LPS-stimulated human peripheral blood mononuclear cells (Yoshimura 1995). These results suggest that cAMP may play an important role in the regulation of TNF-alpha and expression of TNF-alpha mRNA in spleen.

Beta-agonists inhibit the production of TNF-alpha and only partially inhibit IL-1 beta (Yoshimura 1997). This inhibition is through beta-adrenoceptors and elevated

cAMP levels. Many studies have shown that prostaglandins stimulate adenylate cyclase, and PDE inhibitors reduce TNF-alpha steady state mRNA levels in murine peritoneal macrophages or human monocytes, suggesting that beta-agonists also regulate this cytokine at the transcriptional level. With regard to the inhibition of IL-1 production by cAMP-elevating agents, some authors have reported that total IL-1 production is not inhibited in human mixed mononuclear cells or adherent monocytes after treatment with PGE2 or PDE inhibitors. Viherluoto et al (1991) reported, however, that cAMP and PGE2 reduce the amount of secreted IL-1 beta from LPS-stimulated adherent monocytes without affecting the steady-state mRNA levels or cell-associated IL-beta, and that cAMP interferes with secretion of IL-1 beta rather than with other steps in the biosynthetic pathway.

Additional studies support the concept that splenic immune function is under sympathetic control. The in vitro response of splenic lymphocytes to mitogens can be inhibited by catecholamine agonists such as isoproterenol and NE (Goodwin et al 1979). The shock induced suppression of the proliferative response of splenic lymphocytes to ConA can be attenuated by beta-adrenergic receptor antagonists such as propranolol and nadolol, whereas adrenalectomy has been shown to have no effect (Cunnick et al 1988). Together, these findings indicate that ICV PGE2 induced immune suppression of splenic TNF-alpha is mediated by the release of catecholamines into the spleen, and suggest further that the effects of neuronal NE on splenocytes may be direct.

In general, these findings suggest that the sympathetic nervous system may exert a degree of tonic restraint on splenocytes, a conclusion supported by our previous studies

on the sympathetic regulation of splenic macrophage function (Brown et al 1991 and a tendency for splenic denevation to increase the proliferative response to ConA(Wan et al 1993). The present results demonstrate that surgical sympathectomy of the spleen blocks the immunosuppressive effects of ICV PGE2 on the TNF-alpha mRNA expression of splenocytes, suggesting that the splenic nerve transmits immunosuppressive signals to the spleen. The data also suggest further that NE released from nerve terminals are a likely neural mediator of this suppressive effect.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AP-1	Activiting protein-1
cAMP	Cyclic adenosine monophosphate
ConA	Concanavalin A
CRE	cAMP-regulated enhancer
CRF	Corticotrophin-releasing factor
CNS	Central nervous system
EMSAs	Electrophoretic mobility shift assays
HPA	Hypothalamic-pituitary-adrenal
ICV	Intracerebroventricularly
IEGs	Immediate early genes
IL-1	Interleukin-1
IFN	Interferon
IP	Intraperitoneal
LPS	Lipopolysaccharide (endotoxin)
NE	Norepinephrine
NF-AT	Nuclear Factor of Activated T cells
NGS	Normal goat serum
NK	Natural killer
NPY	Neuropeptide Y
Oct-1	Octamer-1

OVLT	Organum vasculosum of the lamina terminalis
MPOA	Medial preoptic area
OT	Oxytocin
SNA	Sympathetic nerve activity
SON	Supraoptic nucleus
SRBC	Sheep red blood cells
PVN	Paraventricular nucleus
PBS	Phosphate buffer saline
PBMCs	Peripheral blood monocytes
PDEs	Phosphodiesterases
PGE2	Prostanglandin E2
irPGE2	Immune reactive prostaglandin E2 type
TNF	Tumor necrosis factor

FIGURE CAPTIONS

Figure 1. Representative photomicrographs illustrating the effects of ICV PGE2 (4 μg) and IV LPS (0.1 μg) on the expression of c-fos protein in the PVN and SON of the hypothalamus. Relative to vehicle-injected controls, there was a dramatic increase in the number of c-fos-positive neurons localized in the PVN and SON following ICV injections of PGE2 and IV injections of LPS. Illustrated in A is the PVN of an animal given a ICV saline. B illustrates that PGE2 injections produced a significant and dramatic increase in the number of c-fos protein positive neurons in the PVN. Illustrated in C is the SON of an anamal given a ICV saline. D illustrates that PGE2 injections produced a significant and dramatic increase in the number of c-fos protein positive neurons in the SON.

Figure 2. Effects of ICV PGE2 following IV LPS on the c-fos protein of PVN. Values are expressed as means (+ SEM) with 10 rats per group. ICV injection of PGE2 produced significantly more c-fos-labeled neurons in the PVN than did ICV saline ($p < 0.001$).

Figure 3. Effects of ICV PGE2 following IV LPS on the c-fos protein of SON. Values are expressed as means (+ SEM) with 10 rats per group. It demonstrates that ICV PGE2 produced significantly more c-fos-labeled neurons in the SON than did saline ICV injections ($p < 0.0001$).

Figure 4. Effects of ICV PGE2 following IV LPS on plasma corticosterone levels. Values are expressed as means (+SEM) with 10 rats per group. Denotes a higher level of plasma corticosterone level difference from saline group, but this difference was not significant (p=0.1282).

Figure 5. This graph demonstrates the effect of the ICV injections of PGE2 on TNF-α mRNA levels relative to saline group, 30 min after ICV injections and one hour after IV LPS. (*) indicates PGE2 group is statistically significant from the saline control (p<0.05).

Figure 6. This graph demonstrates the effect of the ICV injections of PGE2 on IL-1beta mRNA levels, relative to saline group, 30 min after ICV injections and one hour after IV LPS. This was no difference between the PGE2 and saline groups (p=0.8488).

Figure 7. Comparison of c-fos-labeled neurons in the PVN from sham and splenic nerve cut after ICV injections of PGE2 and IV LPS. The two groups did not have difference in terms of the number of c-fos neurons expressed (P=0.3536).

Figure 8. Comparison of c-fos-labeled neurons in the SON from sham and splenic nerve cut after ICV injections of PGE2 and IV LPS. The two groups didn't have difference from each other (P=0.21).

Figure 9. Comparison of plasma corticosterone levels from sham and splenic nerve cut rats after ICV injections of PGE2 and IV LPS. Values are expressed as means (± SEM) with 10 rats per group. This was no statistical difference between splenic nerve cut and sham operated groups (P=0.6638).

Figure 10. The suppressive effect of the ICV injections of PGE2 on IV LPS induced TNF-α mRNA was attenuated in the nerve cut group. Values are expressed as means (± SEM) with 10 rats per group. It showed significantly higher splenic TNF-α mRNA levels than the sham operated group ($p < 0.01$).

Figure 11. This graph demonstrates the effect of the ICV injections of PGE2 on IL-1 β mRNA levels between splenic cut and sham groups, 30 min after ICV injections and one hour after IV LPS. Values are expressed as means (± SEM) with 10 rats per group. The groups were not statistically different (p=0.9326).

Fig.1

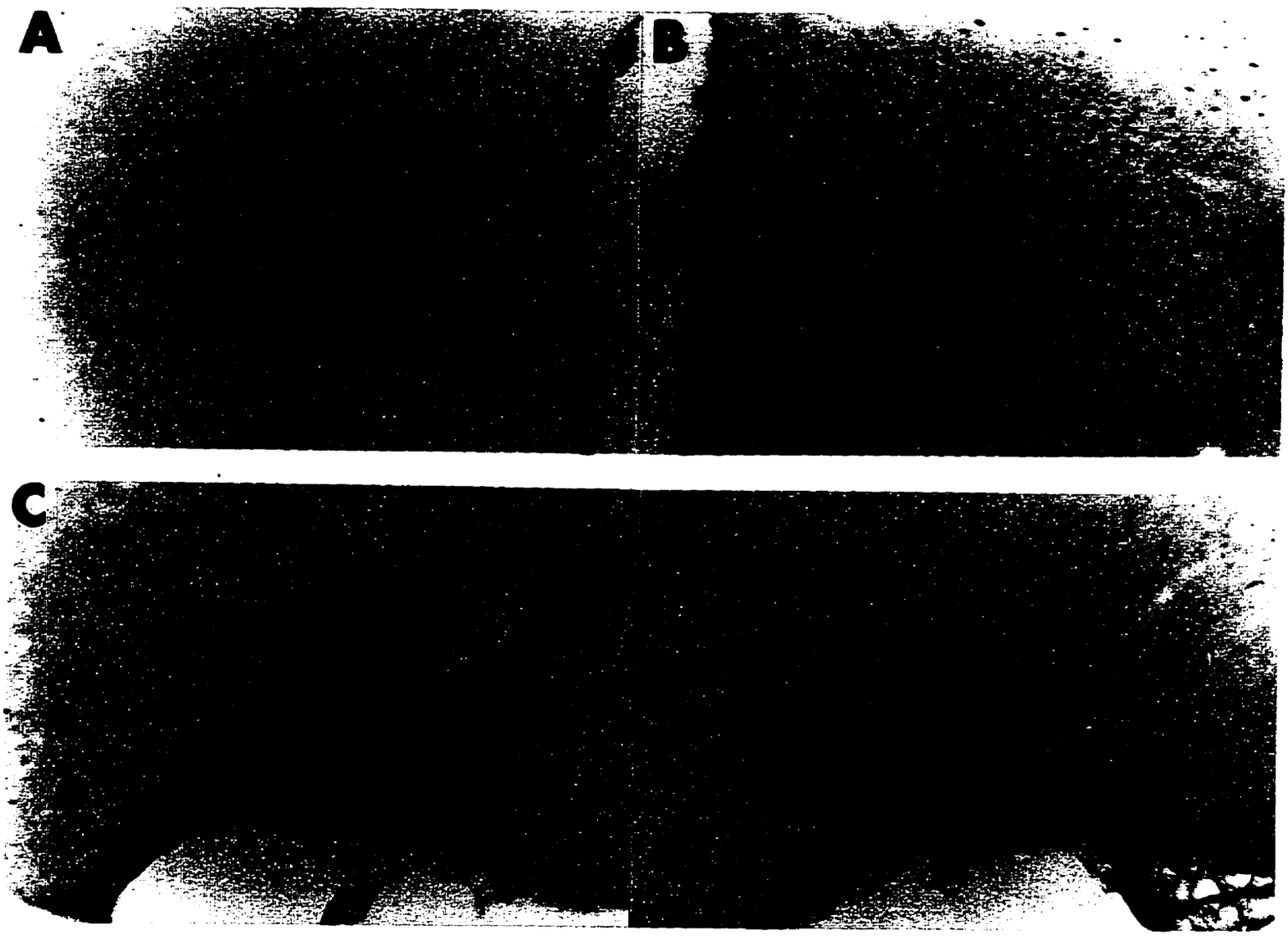


Fig.2

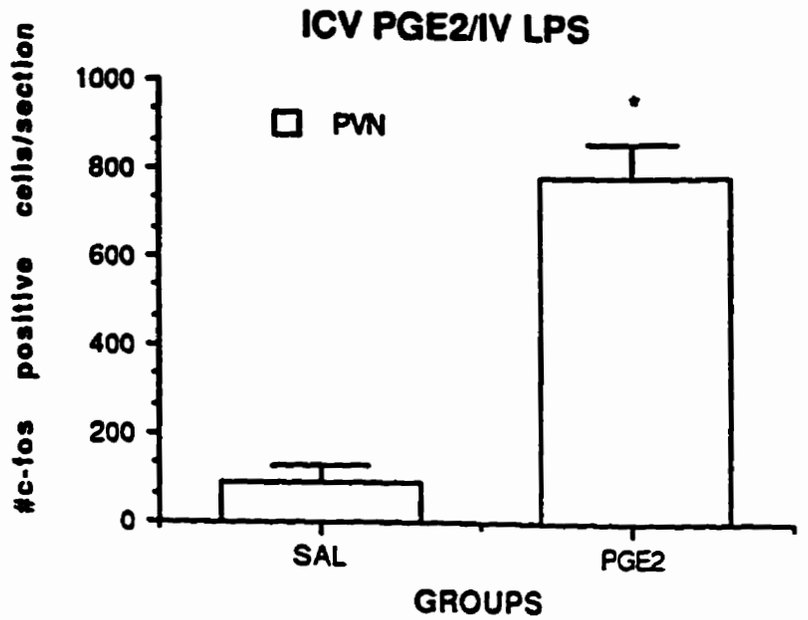

ICV PGE2/IV LPS
#c-fos positive cells/section
1000
800
600
400
200
0
PVN
*
SAL
PGE2
GROUPS

Fig.3

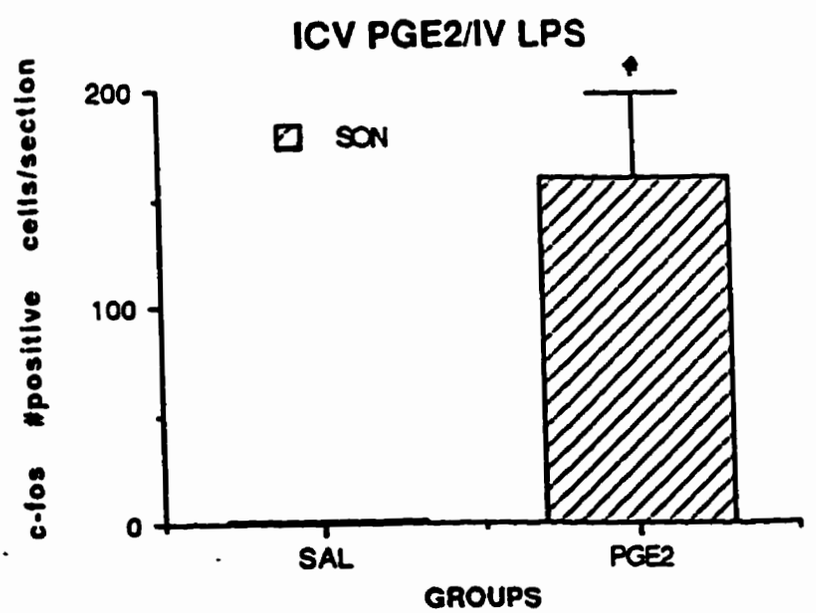

ICV PGE2/IV LPS
c-fos #positive cells/section
200
100
0
SON
*
SAL
PGE2
GROUPS

Fig.4

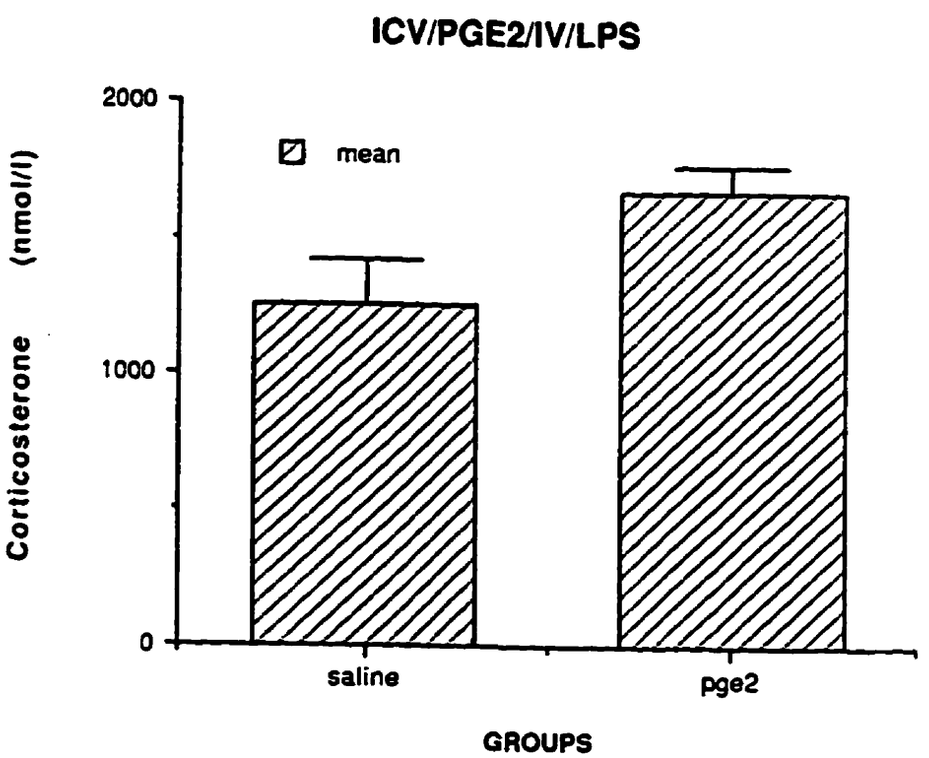

ICV/PGE2/IV/LPS
2000
1000
0
mean
Corticosterone (nmol/l)
saline
pge2
GROUPS

Fig.5

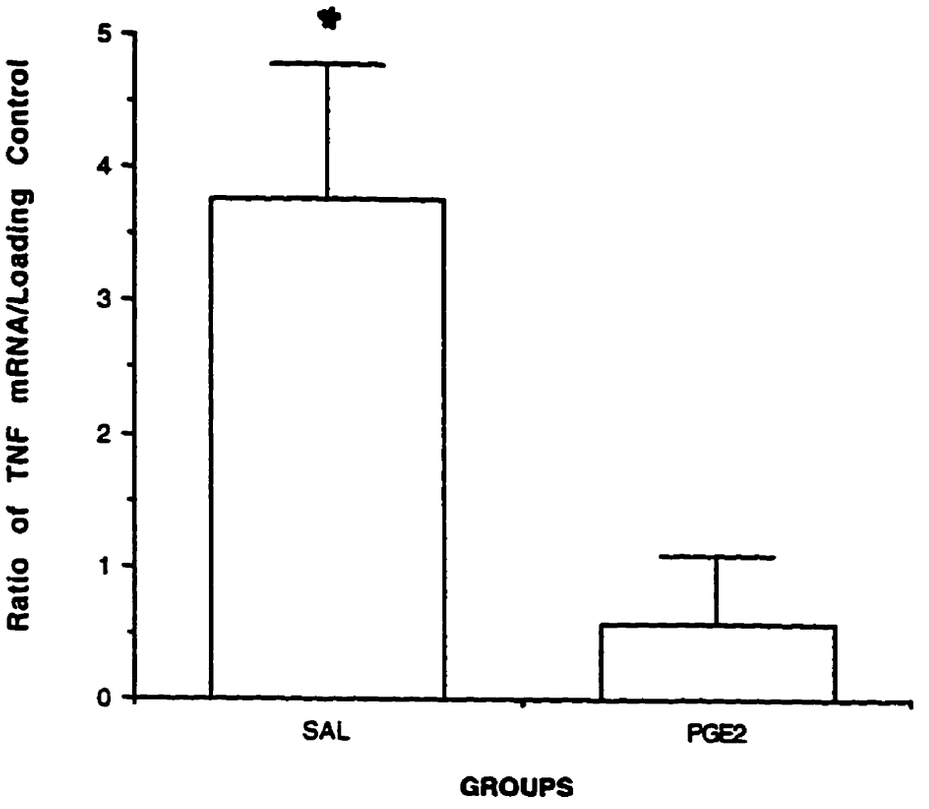


Fig.6

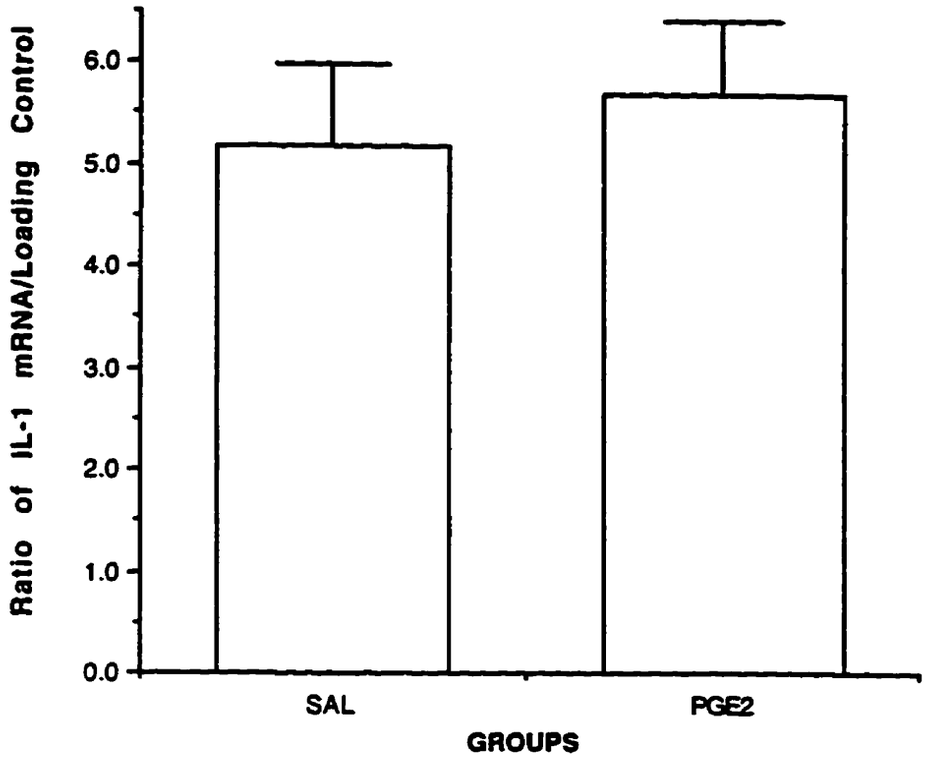

Ratio of IL-1 mRNA/Loading Control
6.0
5.0
4.0
3.0
2.0
1.0
0.0
SAL
PGE2
GROUPS

Fig.7

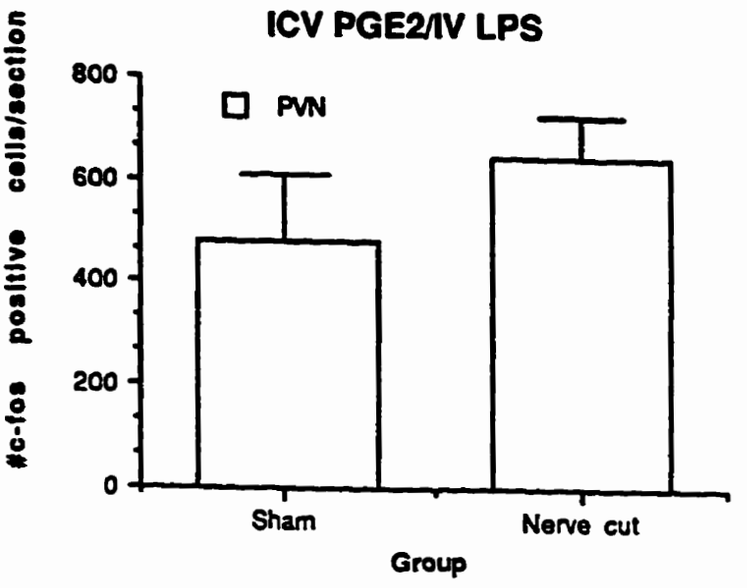

ICV PGE2/IV LPS
PVN
#c-fos positive cells/section
800
600
400
200
0
Sham
Nerve cut
Group

Fig.8

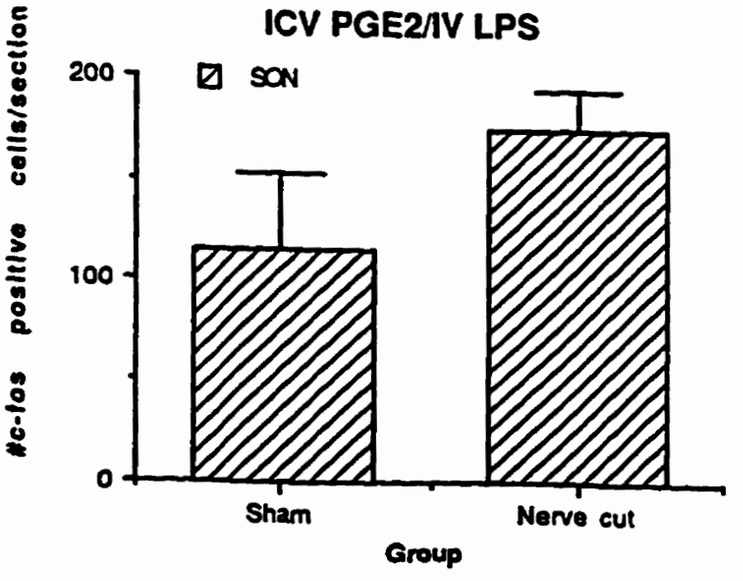

ICV PGE2/IV LPS
SON
#c-fos positive cells/section
200
100
0
Sham
Nerve cut
Group

Fig.9

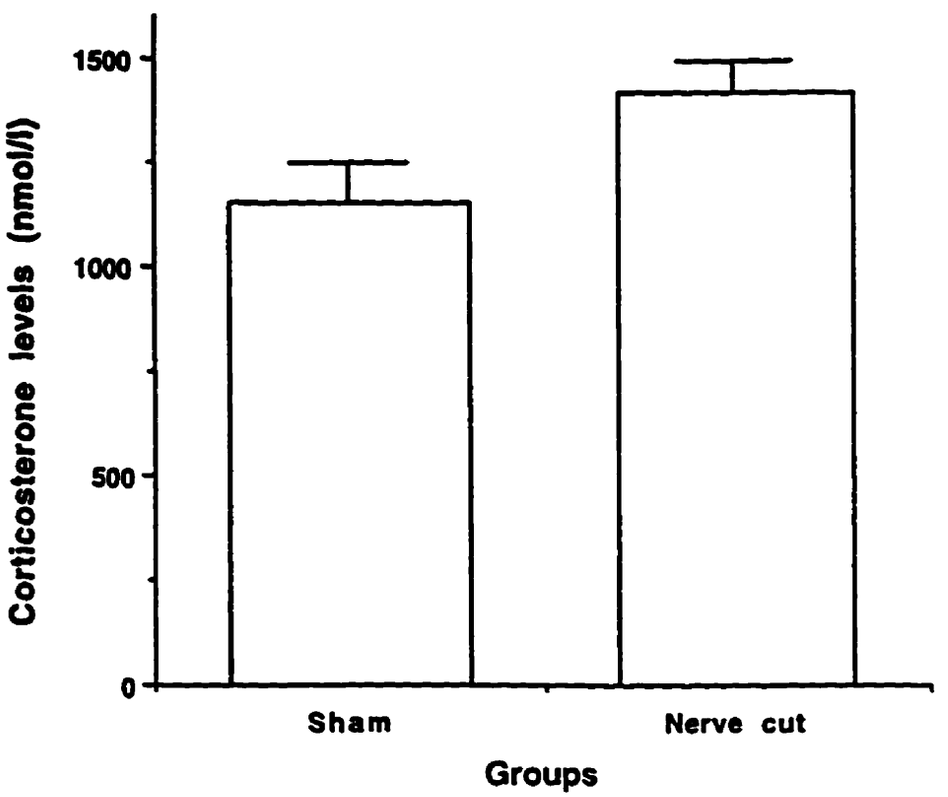

1500
1000
500
0
Corticosterone levels (nmol/l)
Sham
Nerve cut
Groups

Fig.10

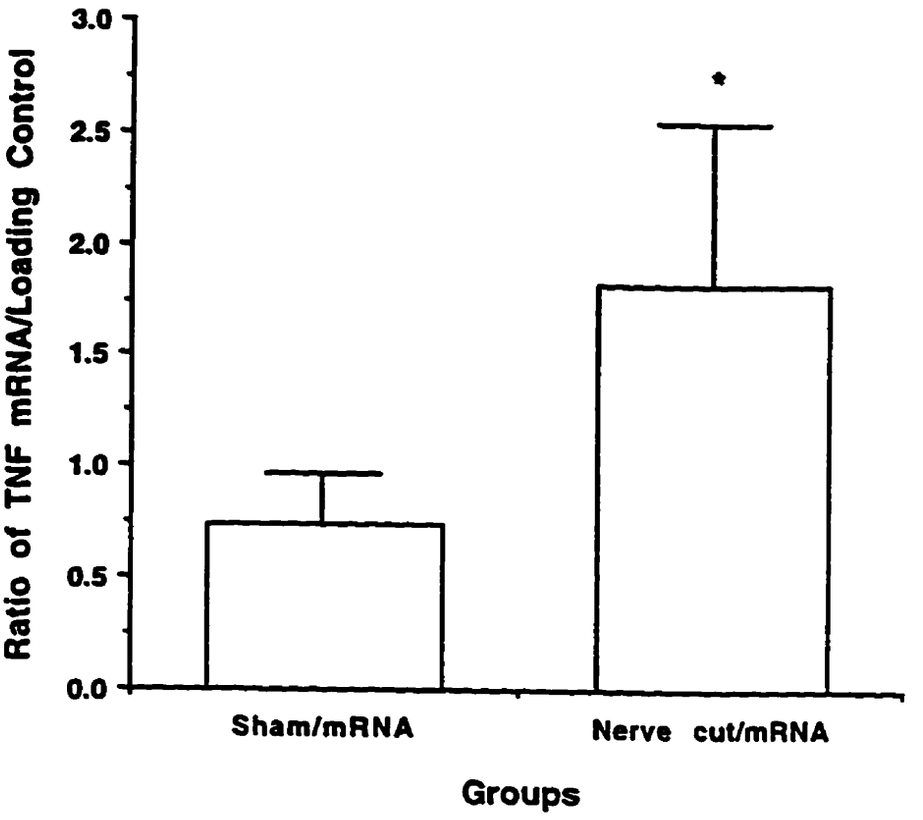
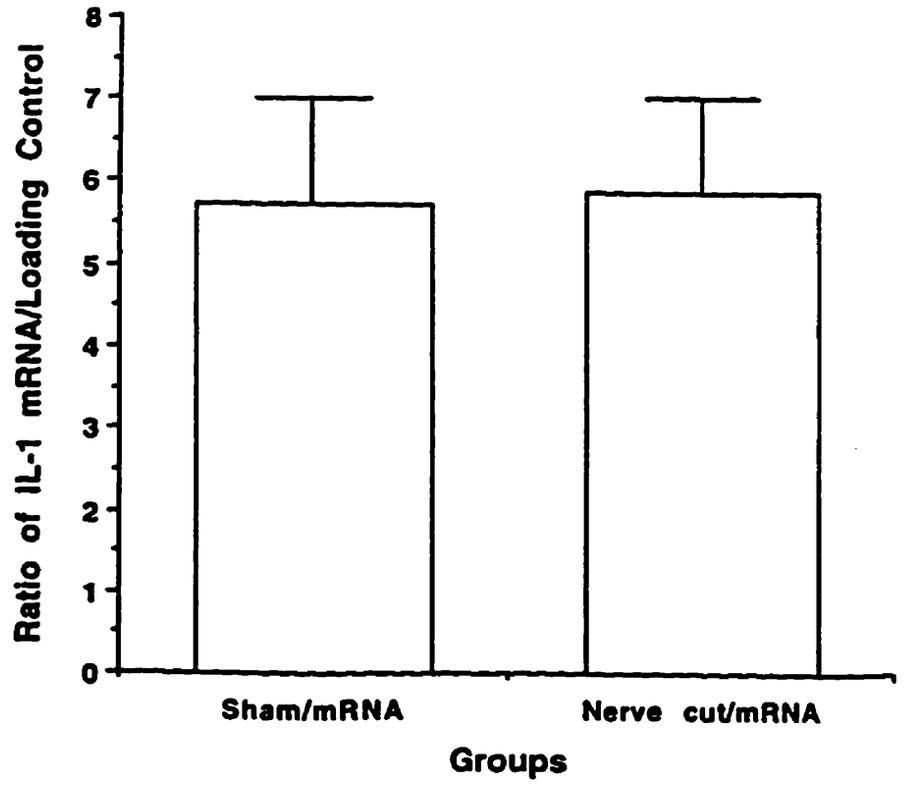

Ratio of TNF mRNA/Loading Control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Sham/mRNA
Nerve cut/mRNA
Groups

Fig.11


Ratio of IL-1 mRNA/Loading Control
8
7
6
5
4
3
2
1
0
Sham/mRNA
Nerve cut/mRNA
Groups