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Mechanisms of Stress-Induced Inhibition of Inflammation

by

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Mechanisms Underlying Stress-Induced Inhibition of Inflammation

Holly J. Strausbaugh

Although largely correlative, a growing body of clinical literature suggests that stress exacerbates signs and symptoms of inflammatory disease. To develop a foundation for the understanding of the complex relationship between stress and inflammatory disease, we investigated the effects of stress on an integral component of the inflammatory response, plasma extravasation. Specifically we investigated whether stress affects plasma extravasation at all and if these effects are generalizable to several stressors. We then investigated both the systemic, neuroendocrine as well as the local, cellular mechanisms of the stress-induced effects on plasma extravasation.

We found that chronic, intermittent stress strongly inhibits neurogenic plasma extravasation while acute stress has no effect. Additionally, we found that these effects are generalizable since three distinct stressors induced them. Our investigation of the neuroendocrine mechanism of these effects indicated that the HPA axis and specifically corticosterone mediates stress-induced inhibition of plasma extravasation. Corticosterone does not, however, inhibit plasma extravasation in a simple, direct way since repeated pulses of corticosterone are required to induce inhibition. Finally, our investigation of the local cellular mechanisms of stress-induced inhibition of plasma extravasation showed that stress-induced inhibition of plasma extravasation is mediated by an effect on neutrophils. The stressful stimulus nearly abolished inflammatory mediator-induced neutrophil accumulation, an event necessary for induction of plasma extravasation, by inducing shedding of L-selectin from circulating

neutrophils. These findings may ultimately contribute to understanding the complex relationship between stress and inflammatory disease.

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CHAPTER ONE

INTRODUCTION

Brief history of the study of stress-induced effects on health

While the belief that stress can have negative health effects is commonly held among the general population, the scientific evidence describing these effects and their mechanisms is quite limited. The concept that stress negatively affects the organism is not a new one in the scientific community however, and was first advanced in 1936 by Hans Selye (Selye, 1936). Seyle's seminal work showed that, in response to diverse stressors including exposure to cold, surgical injury, radiation, exercise and drugs like morphine and atropine animals exhibited a stereotyped response that he termed the "general adaptation syndrome". This syndrome was divided into three stages. The first stage or the alarm reaction occurred 6-48 hours after exposure to the stressor and consisted of a shock phase followed by a countershock phase. In the shock phase animals exhibited a systemic, damaging response to the stressor including tachycardia, decrease in body temperature, decreased thymic weight, development of gastric and intestinal ulcers, increased white blood cell and red blood cell counts, acidosis and decreased blood clotting time (Selye, 1946; Selye, 1936). In the countershock phase he observed reversal of shock phase-associated symptoms. If the stressor was continued, however, the second stage, the resistance phase, ensued. In this phase the adrenal glands became enlarged, the gonads were atrophied and the animal became resistant to further stimulation with the eliciting stressor but became more sensitive to heterotypic stressors. If the stressor exposure continued, after 1-3 months the animals entered the third phase, the exhaustion phase, in which the animal could no longer adapt to the stressor and began to develop "diseases of adaptation". Selye directly observed hypertension, nephrosclerosis and periarteritis nodosa in animals in the exhaustion

phase. Others advanced the idea that due to their time course of development and association with stress, allergies could also be classified as "diseases of adaptation" (Williams, 1945; Williams, 1945). Selye additionally argued that many other diseases including diabetes mellitus, rheumatic fever, thyrotoxicosis, diffuse collagen disease, tonsillitis and appendicitis were also likely to be diseases of adaptation but the empirical evidence was lacking at that time.

Selye's concept of persistent stress-induced diseases of adaptation was met with much criticism (Ingle, 1952; Ingle and Ingle, 1960; Sayers, 1950). His ideas were seemingly completely refuted in 1949 when Hench reported that glucocorticoids, known to be released by stress, were anti-inflammatory and that they specifically could markedly reduce signs and symptoms of rheumatoid arthritis (Hench et al., 1949). Although most investigators agreed with this point of view and numerous studies over the next decades would show a multitude of anti-inflammatory effects of glucocorticoids the evidence that Selye put forth that chronic stress induced harmful physiological changes as well as symptoms of specific disease entities could not be refuted and a handful of investigators continued the pursuit of the investigation of the effects of stress on disease. These studies generally focused on an investigation of the effects of stress on health in humans. To be particularly relevant to humans the studies moved from physical stressors to also include psychological stressors. Many attempts have been made over the last decades to draw a relationship between stress and health in humans. Studies that attempt to correlate major stressful life events with disease episodes generally are retrospective in nature and often do not use control groups. However, several retrospective, controlled studies have demonstrated a

relationship between stressful life events and the onset or relapse of multiple sclerosis (Grant et al., 1989; Warren et al., 1982) rheumatoid arthritis (Henoch et al., 1978; Meyerowitz et al., 1968) diabetes (Kisch, 1985) and inflammatory bowel disease (Duffy et al., 1991) but some have not (Antonovsky et al., 1968; Council, 1950; Pratt, 1951). Some retrospective controlled studies have also demonstrated a positive correlation between incidence of viral upper respiratory infection and life stress (Graham et al., 1986; Jacobs et al., 1970). Even these studies can be difficult to interpret, however, due to their retrospective nature. Specifically, information about stressful life events is usually obtained only from self-reports by patients and it is highly likely that the current disease state of a patient affects their recall of stressful life events. Therefore, drawing conclusions about the relationship between stress and disease in humans based on these types of studies is problematic. Prospective studies are generally not available due to the difficulty in performing such studies. However one rare prospective study investigated the effects of stress on contraction of viral infection (Cohen et al., 1991). This study did show greater incidence of viral infection in persons that had experienced greater life stress during the last year as measured by viral titer as well as by subjective ratings of symptoms (Cohen et al., 1991). Even well designed prospective studies such as these, however, are limited in that it is often difficult to investigate the mechanism of stress effects in humans because of the necessary limits to experimental design.

To be able to investigate the mechanism of stress-induced effects on disease incidence and relapse, investigators have employed the use of experimental animal models. A major limitation of this approach is that the etiology of these diseases is

usually unknown and often expected to be pleiotropic therefore it is often questionable how similar these animal models are to the actual disease. Additionally, many chronic inflammatory diseases have relapsing and remitting courses but the animal models do not relapse and remit making it impossible to use these models to study the effects of stress in evoking relapses. Since many patients report that stress is a major cause of relapse (Affleck et al., 1987) this connection is of great interest for study. Finally, study of mechanism can be difficult even in these animal models because the disease entity is the result of a complex dysregulation of the animal's physiology. It is difficult to understand which part of the complex response may be affected by stress.

To compensate for these limitations, another approach to the study of stress and disease has been to first study the effects of stress on isolated components of the immune response in both animals and humans. In this way complex processes may be broken down and the study of mechanism becomes more feasible. After the mechanisms are understood, this information may then be applied in disease models. Stress has been associated with reliable inhibition of a number of *in vitro* cellular immune parameters including natural killer cell cytotoxicity (Cunnick et al., 1988; Lu et al., 1998), leukocyte proliferation in response to mitogenic stimulation (Dantzer and Mormede, 1995; Keller et al., 1983) and stimulation of leukocyte cytokine production (Dobbs et al., 1996; Glaser et al., 1998; Zwilling, 1994). A limitation to this approach, however, is that this information can be difficult to interpret as it is, as yet, unclear what the impact of discrete changes in isolated immune parameters would be on the general health of an organism. For example, although natural killer cells are known to participate in viral clearance it is unclear if a 40% decrement in their *in vitro* ability to

kill target cells would have any significant *in vivo* impact. For this reason, to move the field forward it has been necessary to adopt *in vivo* approaches in which complex immune responses can be investigated. Some recent studies have successfully employed such approaches and have shown that stress decreases the ability of animals to eradicate HSV-1 virus *in vivo* (Brenner and Moynihan, 1997; Sheridan and Dobbs, 1994) which requires both cellular and humoral immune responses, that stress increases time of wound healing (Padgett et al., 1998) and that stress inhibits antibody production to specific antigens (Glaser et al., 1998; Moynihan et al., 1994). The next step will be to use these models to understand the mechanism of the effects of stress on these complex *in vivo* immune responses and then to finally apply this knowledge to the study of stress and disease.

The aim of the research described herein is to use a similar *in vivo* approach to build a foundation for the future study of the relationship between stress and chronic inflammatory disease by investigating the effects of stress on inflammation. Surprisingly little is known about the effects of stress on inflammation. Inflammation is a complex immune response that represents the first line of host defense against pathogens. Dysregulation of the inflammatory response is thought to underlie the etiology of a host of chronic inflammatory diseases including rheumatoid arthritis, colitis and asthma. Stress has been associated with both incidence and relapse of these diseases (Duffy et al., 1991; Henoch et al., 1978; Meyerowitz et al., 1968; Sekas and Wile, 1980) but there is virtually nothing known about the mechanism of these effects. Therefore understanding how stress affects the inflammatory response and how these effects are mediated would be expected to have a major impact on the understanding

of a host of chronic inflammatory diseases. This information could eventually prove useful in management and perhaps even prevention of chronic inflammatory diseases.

Definition of Stress

To conduct experiments that investigate stress it is first necessary to define it. The difficulty of this task was described by Selye when he explained that "Everybody knows what stress is and nobody knows what it is" (Selye, 1973). A stressful stimulus was formally defined by Selye as "any stimulus to which the organism is quantitatively or qualitatively not adapted" (Selye, 1946). This definition, however, is not readily applied experimentally since it is often difficult to determine the state of adaptation of the animal. Therefore, the widely used operational definition, "any stimulus other than the circadian input to basal activity that excites increased activity in the adrenocortical system" (Dallman, 1991), is the definition that will be used in these studies with the caveat that "...in biology definitions are given merely as concise descriptions of phenomena as they are known at the time, with the view of modifying them as soon as further observations necessitate it" (Selye, 1946). It is important to note that in addition to the adrenocortical system or hypothalamic-pituitary-adrenal (HPA) axis, stressful stimuli activate two other neuroendocrine circuits that may play important roles in stress-induced modulation of inflammation: the sympathoadrenal (SA) axis and the sympathetic efferent axis (Stoddard, 1991) (Figure 1). However, stimuli that activate the hypothalamic-pituitary-adrenal (HPA) axis also activate these other axes to varying degrees (De Boer et al., 1990; Vogel and Jensh, 1988) so the operational definition of stress as a stimulus that activates the HPA axis is an

appropriate definition that encompasses activation of the SA and sympathetic efferent axes.

Overview of the stress axes

HPA Axis

Through a series of steps, stressful stimuli induce activation of the rat HPA axis that results in corticotropin-releasing hormone (CRH)-induced increases in plasma levels of adrenocorticotropin hormone (ACTH) and corticosterone. CRH is released into the portal blood (Plotsky and Vale, 1984) from neurons that project to the median eminence from the paraventricular nucleus of the hypothalamus (Kawano et al., 1988). CRH travels via the portal blood to the anterior pituitary where it induces secretion of ACTH from corticotroph cells into the general circulation (Rivier et al., 1982; Rivier et al., 1982). ACTH acts at the adrenal gland to induce synthesis and secretion of corticosterone from the adrenal cortex. This process is extremely rapid in onset as increased levels of ACTH and corticosterone can be observed in plasma as early as 3 minutes after the onset of stress (Moynihan et al., 1994; Vernikos et al., 1982). ACTH and corticosterone typically remain elevated for 60-120 minutes after the stressor terminates (Bradbury et al., 1991; Gaillet et al., 1991).

SA Axis

Activation of the SA axis by stressful stimuli results in increased plasma levels of epinephrine and opioids that are released from the adrenal medulla. The adrenal medulla also releases low levels of norepinephrine and neuropeptide-Y (NPY),

however, the major source of these latter two mediators is the sympathetic post-ganglionic nerve terminals (see below). Specifically, the release of CRH from autonomic cells of the paraventricular nucleus of the hypothalamus activates sympathetic pre-ganglionic neurons in the spinal cord. These neurons directly innervate the adrenal medulla and induce release from chromaffin cells of granules containing epinephrine and opioids. This process is extremely rapid for epinephrine as increased levels can be observed in plasma as early as one minute after exposure to the stressor (De Boer et al., 1990). Epinephrine levels remain elevated from 10 to 75 minutes after removal of the stressor (De Boer et al., 1990; Zukowska-Grojec et al., 1988). The time course of adrenal medullae derived stress-induced opioid secretion has not been well studied.

Sympathetic Efferent Axis

The sympathetic efferent axis responds to stressful stimuli by releasing norepinephrine and NPY from post-ganglionic nerve terminals. Briefly, autonomic cells of the paraventricular nucleus of the hypothalamus activate sympathetic pre-ganglionic neurons in the spinal cord. These neurons innervate post-ganglionic sympathetic nerves that directly innervate their targets. This process is extremely rapid in onset as increased levels of norepinephrine are observed in plasma as early as one minute after exposure to the stressor (De Boer et al., 1990). They remain elevated from 15 to 75 minutes post stress (De Boer et al., 1990). Elevated levels of NPY are apparent 5 minutes after stressor initiation and remain elevated for 10 minutes after stressor termination (Corder et al., 1992; Zukowska-Grojec et al., 1988). It should be

noted that it is as yet unclear whether stress induces pan-activation of sympathetic post-ganglionic efferents or if specific efferents are selectively activated by stressful stimuli.

Types of Stressors

Animal models of stress have included many different stressors ranging from physical stressors (e.g. hemorrhage, electric shock, cold) to inflammatory stressors (e.g. endotoxin, arthritis) to pharmacological stressors (e.g. ether) to social stressors (e.g. overcrowding, change of cage mate) to psychological stressors (e.g. restraint, exposure to a predator, noise). An ideal stressful stimulus produces reliable activation of the stress axes, is ecologically valid and does not introduce unnecessary confounds into the study. Restraint stress is primarily used in the following body of work because it meets these criteria. First, like all of the stressors listed above, restraint stress evokes reliable activation of the three stress axes (De Boer et al., 1990; Pitman et al., 1988). Second, restraint stress is ecologically valid and does not introduce unnecessary confounds into the study. While many of the other stressors listed above are also ecologically valid, many of them are not appropriate for these studies because they could introduce unnecessary confounds into the study. Microvascular responses in inflammation are measured in these studies, therefore any stressor that directly influences microvascular responses or inflammation introduces a confound because it would be difficult to separate effects of the stressor that are induced by activation of the stress-axis and those that are independent of that activation. For example, cold and exercise stress are not ideal because they exert direct effects on blood flow.

Inflammatory stressors are obviously inappropriate. Social stressors are inappropriate because stressful social encounters usually are accompanied by aggressive behavior that often results in injury to animals in the group (Dantzer and Mormede, 1995) and these injuries elicit an inflammatory response. Restraint stress has few, if any, direct biological effects that would alter components of an inflammatory response independently of stress effects.

Acute and chronic stress

Stress can be administered acutely or chronically. Acute stress is defined as a single exposure of short duration (i.e. less than one hour) to a stressful stimulus (Dallman, 1998). The physiological response to acute stress has been described as "the general alarm reaction" and it is thought to be designed to mobilize the body to respond quickly to a threat (Selye, 1946). Physiological responses to acute stress are relatively consistent across stressors. They include increased circulating levels of ACTH (Akana et al., 1992), glucocorticoids (Akana et al., 1992), epinephrine (De Boer et al., 1990), opioids (De Souza and Van Loon, 1989), norepinephrine (De Boer et al., 1990) and NPY (Zukowska-Grojec et al., 1988). Increases in stress hormones result in increased availability of blood glucose, increased blood supply to skeletal muscle, increased heart rate and blood pressure and increased analgesia (Berne and Levy, 1993). All of these responses return to baseline levels shortly after termination of an acute stress (Akana et al., 1992; De Boer et al., 1990; De Souza and Van Loon, 1989).

Chronic stress is defined as either a series of repeated acute stressors or a continuous stressful stimulus of long duration (greater than one hour) (Dallman, 1998). There is no evidence to date that organisms respond differentially to these two types of chronic stress. The response to chronic stress has been described as the "general adaptation syndrome" and is thought to result in persistent physiological changes that facilitate adaptation to new conditions (Selye, 1946). Some relatively consistent patterns of stress hormone secretion occur after exposure to chronic stress. Levels of corticosterone measured in the diurnal trough (basal levels) are elevated in chronically stressed animals (Ottenweller et al., 1989; Pitman et al., 1988) and basal levels of corticosterone binding globulin (CBG) are decreased (Armario et al., 1994). Additionally, animals that have been chronically stressed tend to display exaggerated ACTH (Akana et al., 1992), corticosterone (Bhatnagar et al., 1995), epinephrine (Konarska et al., 1989) and norepinephrine (Konarska et al., 1989) responses to novel stressors superimposed on the chronic stress (facilitation). Facilitation of NPY and/or opioid release has not yet been reported.

In contrast to increased basal corticosterone, decreased basal CBG and facilitation responses, stress hormone responses measured immediately after exposure to the chronic stressor exhibit much more variability across stressors and individuals. When confronted with a chronic stressor an organism could either continue to respond to the stimulus in the same manner as before (static response), become less responsive to the stressor (habituation) or become more responsive (sensitization). Different studies have demonstrated habituation (Cure, 1989; De Boer et al., 1990), sensitization (Vogel and Jensh, 1988) or a static response (Natelson et al., 1988; Ottenweller et al.,

1989) of plasma levels of ACTH, corticosterone, norepinephrine and epinephrine. Whether the organism responds with habituation, sensitization or a static response appears to be dependent on the nature of the stressor and characteristics of the individual. Some investigators have suggested that the type of response is due to the intensity of the stressor. For example, in the same experiment, rats that were exposed repeatedly to supine restraint showed sensitization of their corticosterone responses while rats that were simply placed in buckets in the same room as the restrained rats showed habituation of their corticosterone responses (Pitman et al., 1988). Other studies suggest that characteristics of the organism determine if it will habituate to a stressor. For example, some animals respond with habituation while others do not, even when exposed to identical stress paradigms in the same study (Natelson et al., 1988; Ottenweller et al., 1989). Understanding the physiological correlates of habituation or sensitization to chronic stressors may provide an explanation of why some individuals are more susceptible to stress-induced disease. Because of the potential relevance of both acute and chronic stress to inflammation both types of stress are investigated in this body of work.

Inflammation

Inflammation is a rapid, protective response to infection or injury that represents a critical element in host defense. The inflammatory response requires the contributions of many cell types (i.e. leukocytes, endothelial cells and neurons) and plasma proteins that act together, in a series of amplified steps. Inflammation is initiated when a substance (e.g. bacteria, chemical irritant) is recognized as "foreign"

by the host. The host can recognize foreign substances specifically or non-specifically. For example, bacterial epitopes can be specifically recognized by T cells and denatured proteins or bacterial cell walls can be recognized non-specifically by the alternative complement pathway or by the contact system. These recognition systems produce pro-inflammatory substances (e.g. interleukin-1, bradykinin, histamine) that continue to amplify the response and induce the characteristic elements of inflammation including extravasation of plasma proteins, recruitment of leukocytes, pain and increased blood flow. Plasma extravasation enables inflammatory mediators and nutritive substances to access the inflammatory site. Additionally, plasma extravasation may help to control inflammation by increasing the rate of removal of tissue-injurious products of the inflammatory response and by increasing extravasation of plasma proteinase inhibitors (e.g., α_1-proteinase inhibitor, α_1-anti-chymotrypsin and α_2-macroglobulin) that control excessive proteolytic activity and thereby protect against connective tissue damage (Kozik et al., 1998). Neutrophils, in the first stages of inflammation, migrate through the endothelium to the site of inflammation where they begin to destroy the pathogen. Pain probably induces the organism to protect the injured area while increases in blood flow allow for faster transportation of leukocytes, and plasma proteins to the site of inflammation.

Plasma extravasation occurs when vasodilating substances (e.g., histamine, platelet activating factor) induced by the initial inflammatory signals bind to endothelial cells. For example, histamine binds directly to endothelial cells and induces actin-mediated formation of gaps up to 1 μm in diameter between cells and subsequent plasma extravasation (Atkinson et al., 1992). The specific mechanisms by

which different inflammatory mediators induce plasma extravasation vary and will be discussed further below for the inflammatory mediators used in these studies.

Neutrophil infiltration into sites of inflammation is mediated by selectins and β_2 integrins. Neutrophils express L-selectin, Mac-1 (CD11B/CD18) and LFA-1 (CD11a/CD18) while endothelial cells express P-selectin, E-selectin, ICAM-1. The initial tethering of neutrophils to and rolling interactions with endothelium are mediated by selectins. Inflammatory mediators (i.e., IL-1, TNF-alpha) induce rapid upregulation of P-selectin expression followed later by upregulation of E-selectin expression on endothelial surfaces. L-selectin is constitutively expressed on neutrophils and rapidly shed upon their activation. Recent studies investigating P- and L- selectin knockout mice indicate that P-selectin is required early in the rolling process while L-selectin is required later (Ley et al., 1995). Firm adhesion is mediated by the β_2 integrins, LFA-1 and Mac-1, on neutrophils and their ligand ICAM-1 on endothelial cells. Inflammatory mediators induce upregulation of ICAM-1 expression on endothelial cells. Chemoattractants (e.g., interleukin-8) produced at the inflammatory site induce more adhesive conformations of LFA-1 and Mac-1. After firm adhesion, shape change and diapedesis occurs through mechanisms involving chemokines and their receptors that are not well-understood.

Neutrophil adhesion may also contribute to plasma extravasation. For example, several studies have shown that inhibiting neutrophil recruitment at sites of inflammation significantly blocks plasma extravasation (Bjerknes et al., 1991; Bjork et al., 1982; Green et al., 1993; Wedmore and Williams, 1981). Additionally, catecholamine antagonists and agonists exert much less pronounced effects on plasma

extravasation if neutrophils are inhibited from adhering to inflammatory sites (Weisdorf and Jacob, 1987). The mechanism by which neutrophils influence plasma extravasation is not known but it is known that it does not involve superoxide release from neutrophils (Rosengren et al., 1988).

Inflammatory pain is induced by sensitization of nociceptive afferents by inflammatory mediators (Martin et al., 1987; Taiwo et al., 1987). Increased blood flow occurs primarily due to vasodilation of resistance vessels. This occurs when vasodilating substances like histamine, bradykinin (BK) and substance P bind directly to endothelial cells. This induces release of nitric oxide and subsequent relaxation of vascular smooth muscle cells which results in vasodilation and increased blood flow. Production and release of the vasodilating substances is induced by the initial inflammatory signals.

Stress and Inflammation

Very little is known about the effects of stress on the inflammatory response. Human studies have drawn correlations between inflammatory disease activity and psychological stress. For example, it has been shown in patients that mild daily stressors exacerbate signs and symptoms of rheumatoid arthritis (Potter and Zautra, 1997; Zautra et al., 1994), ulcerative colitis (Levenstein et al., 1994) and inflammatory bowel disease (Dancey et al., 1998). Additionally, higher levels of perceived stress are associated with increased incidence of asthmatic episodes (Sekas and Wile, 1980). Both patients with rheumatoid arthritis (Affleck et al., 1987) and irritable bowel syndrome (Robertson et al., 1989) cite stress as the most frequent cause of symptom

flares and stress management interventions can reduce these symptom flares in rheumatoid arthritis patients (Parker et al., 1995). Little is known about the nature of the stressors in these studies since it is difficult to isolate specific stressors and their characteristics when the experiments use naturally occurring stressors in the subjects' daily lives. However, in one rare study that did attempt to investigate the nature of the stressors involved, Potter et al. (Potter and Zautra, 1997) found that mild daily life stress is highly correlated with symptom flares in inflammatory disease while major life stresses are actually negatively correlated with flares. Although suggestive, it must be noted that these studies are limited in that it is impossible to determine whether stress modulated disease activity or if increased disease activity induced increased symptoms that resulted in increased stress.

Information from animal studies investigating the effects of stress on inflammation is limited. Acute stress decreases the plasma extravasation response to inflammatory stimuli (Bhattacharya et al., 1987; Harmsen and Turney, 1985). Additionally, the only study to investigate the effects of stress on neutrophil function demonstrated that stress inhibits inflammatory stimulus-induced neutrophil accumulation *in vivo* but increases neutrophil adherence to plastic *in vitro* (Harmsen and Turney, 1985). Only one study has investigated the effects of chronic stress on any component of the inflammatory response. Specifically, Padgett et al. (Padgett et al., 1998) have shown that chronic restraint stress decreases cellular infiltrate 1 and 3 days after initiation of the wound in a model of cutaneous wound healing. No reported studies have investigated the effects of stress on the inflammatory response

continuously over a substantial time period. Additionally, there is very little information available about the mechanisms of the reported stress-induced effects.

Some studies have investigated the effects of specific stress hormones on components of the inflammatory response. These studies are briefly reviewed below.

HPA Axis and Inflammation

Glucocorticoids and Inflammation

Glucocorticoids have been widely used clinically to treat inflammatory diseases. In studies that investigate the therapeutic effects of these treatments, glucocorticoids are effective in reducing disease activity at least for some portion of the patients studied (foundation, 1959; foundation, 1960; Lennard-Jones et al., 1960; Taylor and Shaw, 1993; Truelove and Witts, 1954). However, the effects of glucocorticoids on inflammatory disease activity are likely to be complex for some inflammatory diseases. For example, as a treatment for rheumatoid arthritis, low dose glucocorticoid therapy only appears to be marginally effective (Saag et al., 1996) and even in some studies that use higher doses, a relatively large number of patients (40%) do not respond to glucocorticoid therapy (van Gestel et al., 1995). Additionally, in a study that showed a positive correlation between daily stressful events and rheumatoid arthritis flares, plasma cortisol was *positively* associated with rheumatoid arthritis flares (Zautra et al., 1994).

Glucocorticoids exert many of their anti-inflammatory effects by inhibiting production of pro-inflammatory cytokines including TNF-α (Calandra et al., 1995), interleukin-1β (Snyder and Unanue, 1982), interleukin-2 (Gillis et al., 1979; Gillis et

al., 1979), interluekin-6 (Calandra et al., 1995), interleukin-8 (Calandra et al., 1995) and interferon gamma (Rytel and Kilbourne, 1966) as well as other inflammatory mediators like prostaglandins and leukotrienes (Blackwell et al., 1978). Glucocorticoids inhibit cytokine production by suppressing cytokine gene expression. Glucocorticoids suppress cytokine gene expression both by repressing the transcription factor AP-1 (Diamond et al., 1990) and by inducing synthesis of the inhibitory protein IκBα which represses the transcription factor NF-κB (Auphan et al., 1995; Scheinman et al., 1995). Glucocorticoids inhibit prostaglandin and leukotriene production by induction of lipocortin. Lipocortin inhibits the synthesis pathway of prostaglandins and leukotrienes early on by inhibiting the activity of phospholipase A2, the enzyme that acts on arachidonic acid to begin synthesis of prostaglandins and leukotrienes (Blackwell et al., 1982; Blackwell et al., 1980).

Glucocorticoids also affect plasma extravasation. For example, dexamethasone decreases plasma extravasation in a receptor-mediated fashion in an air pouch model of inflammation (Laue et al., 1988). Additionally, lipocortin, which is induced by corticosterone, inhibits synovial plasma extravasation (Green et al., 1998).

Glucocorticoids may also modulate neutrophil recruitment to inflammatory sites. Corticosterone inhibits accumulation of leukocytes at inflammatory sites (Yarwood et al., 1993). Additionally, dexamethasone induces neutrophilia in the blood (Mishler and Emerson, 1977) which may be due, at least in part, to the inability of neutrophils to migrate out of the blood since dexamethasone decreases neutrophil adhesion to cultured endothelium (Cronstein et al., 1992; Schleimer et al., 1989).

However, other studies show that *in vitro* treatment of neutrophils with dexamethasone does not inhibit adhesion to endothelial cells (Diaz-Gonzalez et al., 1995). Other studies corroborate that glucocorticoids inhibit leukocyte emigration from blood (Mancuso et al., 1995; Oda and Katori, 1992; Tailor et al., 1997) but indicate that glucocorticoids do not inhibit leukocyte adhesion and rolling (Mancuso et al., 1995; Oda and Katori, 1992) or that glucocorticoids only inhibit leukocyte adhesion and rolling at very high doses (Tailor et al., 1997).

Glucocorticoids could affect neutrophil recruitment by modulating the expression of adhesion molecules on neutrophils (i.e., L-selectin, PSGL-1, CD11a/CD18, CD11b/CD18), adhesion molecules on endothelium (i.e., E-selectin, ICAM-1, ICAM-2, P-selectin). The effect of glucocorticoids on L-selectin expression is unclear. *In vivo* treatment with glucocorticoids (Burton et al., 1995; Jilma et al., 1997) decreases L-selectin expression on neutrophils, however, *in vitro* studies have failed to demonstrate an effect of glucocorticoids on L-selectin expression by resting neutrophils (Diaz-Gonzalez et al., 1995; Filep et al., 1997) but dexamethasone inhibits PAF-induced shedding of L-selectin and concomitant upregulation of β_2 integrins on neutrophils *in vitro* (Filep et al., 1997). Only one study has investigated the effects of *in vivo* glucocorticoid treatment on β_2 integrin expression on neutrophils. In that report, both dexamethasone and cortisol induced persistent increases in CD18 expression on neutrophils (Burton et al., 1995). The effects of glucocorticoids on the expression of endothelial adhesion molecules have not been well-studied. One report, however, suggests that corticosterone decreases the expression of ICAM-1 and E-

selectin in a glucocorticoid receptor-mediated fashion (Cronstein et al., 1992). The effects of glucocorticoids on ICAM-2 and P-selectin have not been reported.

Glucocorticoids ultimately decrease inflammatory pain because they inhibit synthesis of inflammatory mediators (e.g., leukotrienes, prostaglandins) (Blackwell et al., 1982; Blackwell et al., 1980) that sensitize nociceptive afferents (Martin et al., 1987; Taiwo et al., 1987). Glucocorticoids exert indirect effects on blood flow since they are permissive for vasoconstrictive actions of epinephrine and norepinephrine (Berne and Levy, 1993).

Most of the effects reviewed here regarding glucocorticoids and inflammation indicate that glucocorticoids suppress inflammatory responses. It is important to note, however, that glucocorticoids may have a complex dose response curve when it comes to these actions. For example, it has recently been shown that low doses of glucocorticoids, as well as mild stress, induce expression of the pro-inflammatory cytokine, macrophage migration inhibitory factor (MIF), while higher doses of glucocorticoids do not (Calandra et al., 1995). MIF antagonizes the ability of glucocorticoids to downregulate expression of TNF-α, interleukin-1β, interleukin-6 and interleukin-8 indicating that at these doses glucocorticoids are pro-inflammatory with respect to production of these pro-inflammatory cytokines (Calandra et al., 1995). The effects of MIF on other inflammatory effects of glucocorticoids have not yet been investigated.

Although glucocorticoids exert profound effects on components of the inflammatory response, it is likely that other stress-induced hormones also affect inflammation. For example, literature on the effects of stress on other immune

responses demonstrates that although glucocorticoids mediate some stress-induced effects, others are completely independent of glucocorticoids and are instead mediated by the SA and sympathetic efferent axes (Dobbs et al., 1993; Irwin and Hauger, 1988; Keller et al., 1983).

ACTH and CRH and Inflammation

CRH has been found in inflamed joints of both humans (Crofford et al., 1993) and rats (Crofford et al., 1992) with rheumatoid arthritis and in animal models of acute, non-synovial inflammation (Karalis et al., 1991). It appears that this CRH is being secreted locally as these increased levels do not correspond with increases of CRH in systemic circulation. CRH may be released by local cells as CRH mRNA and protein have been identified in leukocytes (Stephanou et al., 1990). Investigations of the role of CRH at local inflammatory sites reveal that CRH inhibits plasma extravasation (Karalis et al., 1991; Serda and Wei, 1991) and that this effect is independent of steroid release and blocked by a CRH antagonist (Karalis et al., 1991). CRH has also been shown to decrease neutrophil infiltration into inflamed peritoneum (Karalis et al., 1991). CRH injected into inflammtory sites induces analgesia (Hargreaves et al., 1989; Schafer et al., 1994). However, this effect appears to be mediated by CRH-induced release of opioids from leukocytes (Kavelaars et al., 1990; Schafer et al., 1994) since there are no CRH receptors on sensory neurons (Mousa et al., 1996), there are CRH receptors on leukocytes (Karalis et al., 1991; Stephanou et al., 1990) that are upregulated during inflammation (Mousa et al., 1996) and CRH-induced analgesia is blocked by opioid antagonists (Schafer et al., 1994). CRH also

affects blood flow as peripheral injection of CRH induces hypotension (Corder et al., 1992; Lei et al., 1993). Whether the described effects of local CRH are relevant to the investigation of stress and inflammation remains an open question since it is unclear whether stress can induce local release of CRH and CRH released from central sites does not reach appreciable levels in plasma.

Very little is known about the role of ACTH in inflammation. A role for ACTH at inflammatory sites has been postulated. Specifically, it was shown that ACTH is present in lymphocytes (Smith et al., 1986) and that its expression could be induced by the potent inflammatory stimulus, lipopolysaccharide (Harbour et al.,1987). However, since these initial observations, other investigators have not been able to detect ACTH in lymphocytes (van Woudenberg et al., 1993) and those that have detected it have shown that these ACTH transcripts are not properly processed resulting in a truncated form that is not secreted (Clark et al., 1990). ACTH effects on other components of the inflammatory response, independent of its function as a glucocorticoid secretagogue, have not been reported.

SA Axis and Inflammation

Epinephrine and Opioids and Inflammation

Epinephrine inhibits plasma extravasation (Coderre et al., 1991). Although there is little data available on the effects of epinephrine on neutrophil recruitment one study reports that *in vitro* treatment with epinephrine decreases adhesion of fMLP-treated neutrophils to endothelial monolayers (Bazzoni et al., 1991). Epinephrine increases pain by sensitizing nociceptors (Khasar et al., in press) and epinephrine

exerts profound effects on blood flow by constricting arterioles that supply vascular beds (Berne and Levy, 1993).

Opioids inhibit plasma extravasation (Green and Levine, 1992; Khalil and Helme, 1990) while the effect of opioids on neutrophil recruitment is unclear. There is some suggestive evidence that opioids may affect neutrophil recruitment by affecting both neutrophil and endothelial cells, however, the data are limited and sometimes contradictory. *In vitro* studies indicate that opioids increase neutrophil adhesion to serum-coated glass (Van Epps and Kutvirt, 1987) and to endothelial monolayers (Fischer et al., 1990), increase spreading (Falke and Fischer, 1985), decrease Mac-1 expression and inhibit neutrophil aggregation (Mazzone et al., 1990). Additionally, treatment of endothelial cell monolayers with opioids promotes neutrophil migration across them to a similar degree as TNF-α (Wiedermann et al., 1994). Opioids have profound analgesic effects and opioids are known to induce vasodilation (White et al., 1990) but it is unclear whether this is due to direct effects on the endothelium or if vasodilation is mediated by secondary factors (Karasawa et al., 1993).

Sympathetic Efferent Axis and Inflammation

Norepinephrine and Neuropeptide Y and Inflammation

Alpha$_2$-adrenergic agonists increase BK-induced plasma extravasation while alpha$_2$-adrenergic antagonists inhibit it (Coderre et al., 1991). Norepinephrine binds to alpha$_2$-adrenergic receptors with high affinity. Effects of norepinephrine on neutrophil recruitment have not been reported, however, epinephrine decreases adhesion of

fMLP-treated neutrophils to endothelial monolayers via its action at β-adrenergic receptors (Bazzoni et al., 1991). This finding may be relevant to norepinephrine effects since norepinephrine binds to β-adrenergic receptors with low affinity. It is possible that the levels of norepinephrine released during stress are high enough that this binding becomes functionally relevant. Additionally, since glucocorticoids increase the number of beta-adrenergic receptors on neutrophils *in vivo* (Davies and Lefkowitz, 1980) and increase their coupling to adenylate cyclase (Davies and Lefkowitz, 1981) both *in vitro* and *in vivo*, during stress when plasma glucocorticoid levels are high, norepinephrine may exert important effects at these receptors. Norepinephrine induces hyperalgesia by an indirect action on sensory neurons (Levine et al., 1986; Taiwo et al., 1987). Like epinephrine, norepinephrine is a potent vasoconstrictor that decreases blood flow to vascular beds by constricting arterioles that supply these beds (Berne and Levy, 1993).

NPY inhibits plasma extravasation (Levine, unpublished data). There is only one reported study addressing the effects of NPY on neutrophil recruitment. Sung et al. showed that treatment of endothelial monolayers with NPY increased adhesion of neutrophils to the monolayers and that this increased adhesion was not mediated by effects on ICAM-1 expression (Sung et al., 1991). NPY effects on pain have not been reported. NPY decreases blood flow by its vasoconstrictive actions as well as by its ability to potentiate the vasoconstrictive actions of epinephrine and norepinephrine (Zukowska-Grojec et al., 1986).

Knee Joint Model of Inflammation

In the following studies, knee joint plasma extravasation, in response to inflammatory mediators, is used as the model of inflammation. Synovial plasma extravasation is a good inflammatory model to use for these studies for the following reasons: 1.) Plasma extravasation is an integral component of the inflammatory response, therefore it is a valid measure of inflammation. 2.) Synovial plasma extravasation is an *in vivo* model in which inflammation is measured in an intact organism. Much of the existing literature on stress and inflammation is based on *in vitro* results and when *in vivo* data is available many contradictions exist between *in vitro* and *in vivo* results. Use of this model may help to resolve these contradictions. 3.) This model allows for continuous monitoring of the plasma extravasation response over time. Therefore a more complete picture of the inflammatory response can be obtained. 4.) Use of the synovial space is ideal since chronic inflow needles permit easy administration of inflammatory mediators as well as antagonists and agonists to potential mediators at any time during the inflammatory response. 5.) Synovial plasma extravasation has been associated with experimental arthritis therefore hypotheses generated from these data could be directly relevant to experimental arthritis.

In these studies, bradykinin (BK) or platelet activating factor (PAF) are administered into the synovial space as inflammatory stimuli. BK is an endogenous inflammatory mediator that stimulates an inflammatory response composed of all the principal components of inflammation including plasma extravasation, infiltration of leukocytes, sensitization of primary afferent nociceptors and vasodilation. The exact mechanism by which BK induces plasma extravasation is unknown (Figure 2),

however it is know that the presence of post-ganglionic sympathetic nerve terminals is required and therefore BK-induced plasma extravasation is considered a model of *neurogenic* inflammation. Interestingly, activity in these neurons is not required for BK-induced plasma extravasation. It is likely that BK elicits factors known to be involved in BK-induced plasma extravasation (e.g., prostaglandins, ATP, adenosine) (Green et al., 1993) directly from these terminals independent of neural transmission (Green et al., 1997). The presence of BK receptors on these terminals is supportive of this model (Boehm and Huck, 1997; Seabrook et al., 1997). Sensory neurons and/or the presence of mast cells are not required for BK-induced synovial plasma extravasation. Elicitation of BK-induced plasma extravasation also requires the presence of neutrophils. The neutrophil requirement for plasma extravasation induction has been observed by others in different inflammatory models (Bjork et al., 1982; Wedmore and Williams, 1981) but the nature of their contribution is unclear. It is known, however, that the mechanism by which neutrophils influence plasma extravasation does not involve superoxide release from neutrophils (Rosengren et al., 1988). Neutrophils may contribute to BK-induced plasma extravasation by supplying nitric oxide which is involved in the mechanism of BK-induced plasma extravasation (Green et al., 1993). The mechanism of plasma extravasation induced by the endogenous inflammatory mediator, PAF, is also unknown (Figure 2). It is known that PAF induces plasma extravasation by a different mechanism than BK that does not involve the presence of sympathetic post-ganglionic efferents (Green et al., 1993) or sensory neurons therefore PAF-induced plasma extravasation is considered a model of *non-neurogenic* inflammation. PAF-induced plasma extravasation does not require

the presence of neutrophils and it is thought that PAF may induce plasma extravasation by a direct action mediated through its receptors on endothelial cells (Green et al., 1993).

Figure 1. Schematic of the three stress-activated neuroendocrine circuits.

Stress induces activation of the HPA axis, the SA axis and the sympathetic efferent axis. HPA axis activation is induced when CRH is released into the portal blood (Plotsky and Vale, 1984) from neurons that project to the median eminence from the paraventricular nucleus of the hypothalamus (Kawano et al., 1988). CRH then evokes ACTH release from the anterior pituitary. ACTH travels through the circulation to the adrenal gland where it stimulates synthesis and release of corticosterone. The sympathoadrenal axis is activated when release of CRH from autonomic cells of the paraventricular nucleus of the hypothalamus activates sympathetic pre-ganglionic neurons in the spinal cord. These neurons directly innervate the adrenal medulla and induce release of granules containing epinephrine and opioids from chromaffin cells. Peripheral sympathetic outflow is induced by stress when autonomic cells of the paraventricular nucleus of the hypothalamus activate sympathetic pre-ganglionic neurons in the spinal cord. These neurons innervate post-ganglionic sympathetic nerves that directly innervate their targets. These peripheral sympathetic nerves release norepinephrine and NPY upon activation.

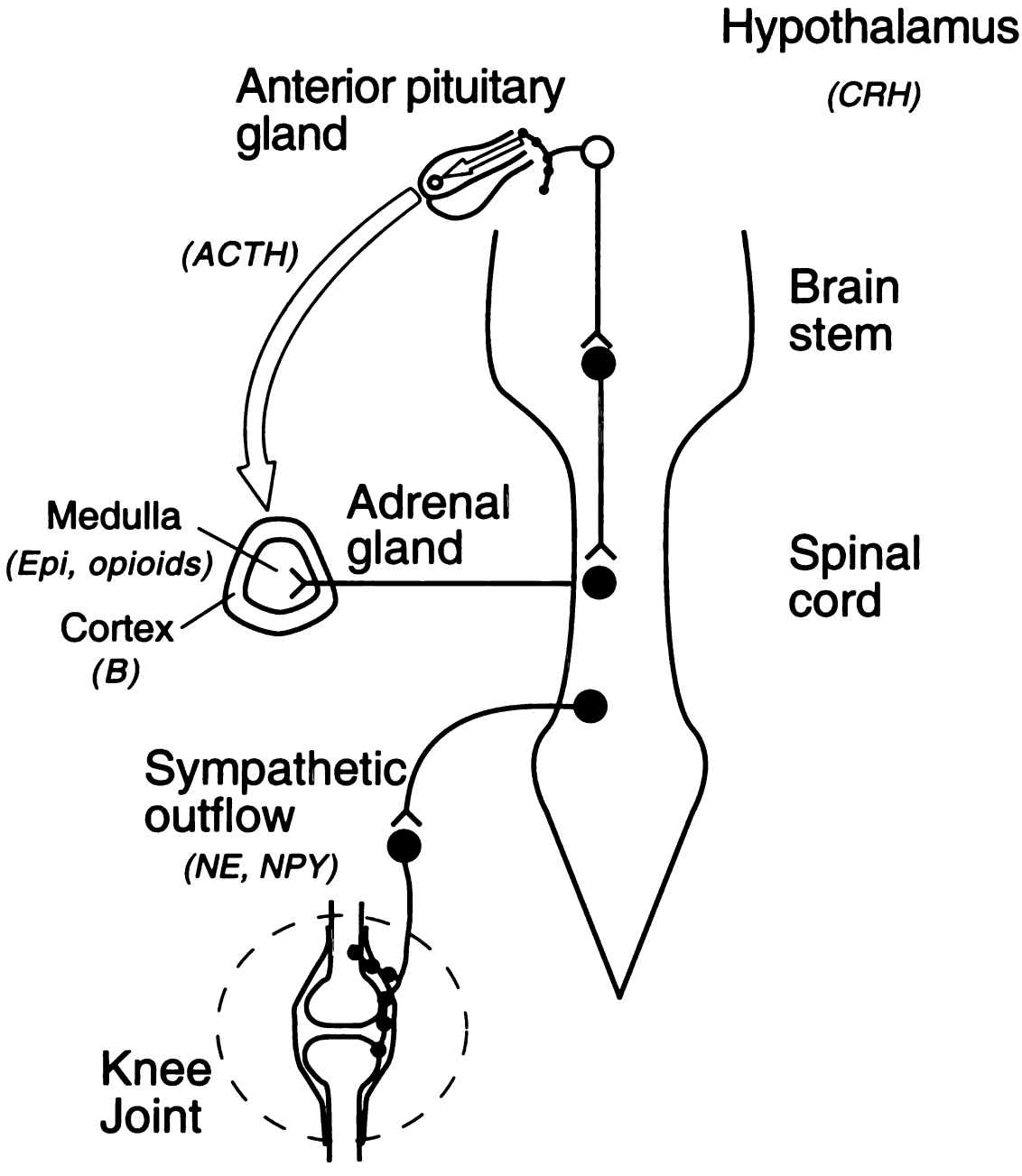
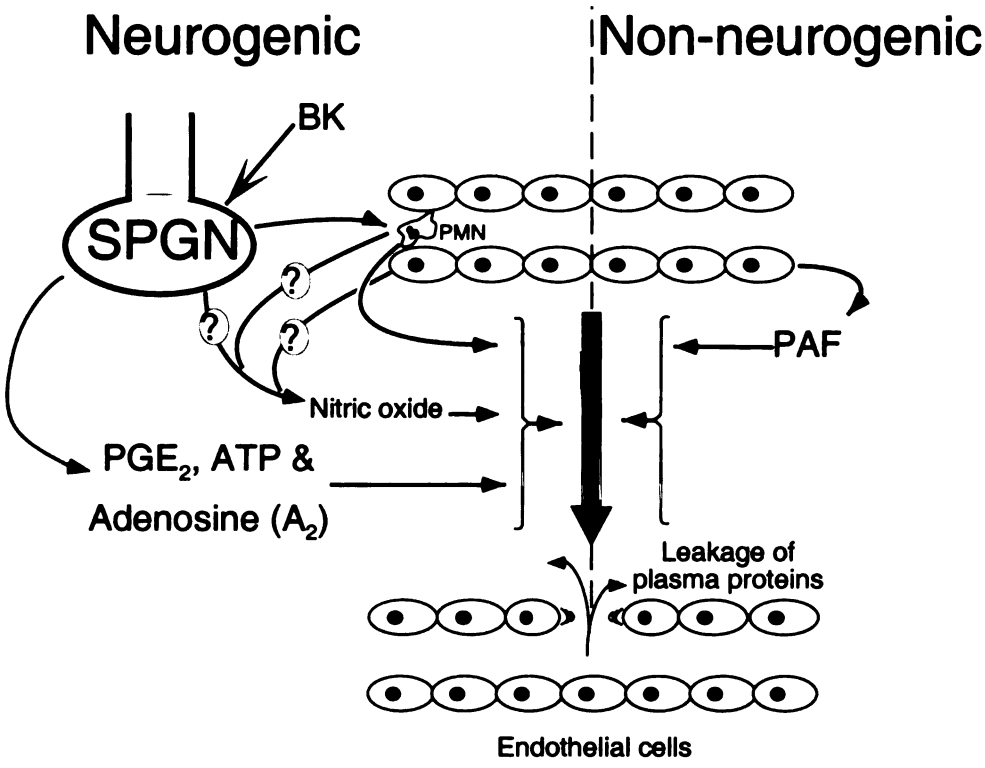

Hypothalamus
(CRH)
Anterior pituitary gland
(ACTH)
Brain stem
Medulla
(Epi, opioids)
Adrenal gland
Cortex
(B)
Spinal cord
Sympathetic outflow
(NE, NPY)
Knee Joint

Figure 2. Putative mechanisms of BK- and PAF-induced plasma extravasation.

BK-induced plasma extravasation requires the presence of neutrophils and sympathetic post-ganglionic nerve terminals. The nature of the contribution of theses cells is unknown, however, they may provide plasma extravasation-promoting factors (e.g., nitric oxide, prostaglandins, ATP, adenosine). PAF-induced plasma extravasation does not require the presence of neutrophils or sympathetic post-ganglionic nerve terminals. While the mechanism of PAF-induced plasma extravasation is also unknown, it is thought that PAF induces PE by a direct action on endothelial cells.


Neurogenic
Non-neurogenic
BK
SPGN
PMN
?
?
?
PAF
Nitric oxide
PGE2, ATP &
Adenosine (A2)
Leakage of
plasma proteins
Endothelial cells

CHAPTER TWO

GENERAL METHODS

Animals

The experiments were performed on 300-400 g male, Sprague-Dawley rats (Bantin and Kingman, Fremont, CA). Care and handling of animals were performed in accordance with the American Physiological Society guidelines. The experimental protocols were approved by the UCSF Committee on Animal Research.

Plasma extravasation

Procedure

As described previously (Coderre et al., 1989), rats were anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneal) and were then given a tail vein injection of Evans blue dye (50 mg/kg in a volume of 2.5 ml/kg) which binds stoichiometrically to serum albumin. Fluid was perfused through the knee joint at a constant rate (250 µl/min) and perfusate samples were collected every 5 minutes for a period of 75-90 minutes (Figure 3). After establishing baseline levels of plasma extravasation in the first three samples, the inflammatory mediator (BK; 150 nM or PAF 35nM) was added to the perfusing fluid (normal saline for BK, 0.2% bovine serum albumin in normal saline for PAF) and remained present in the fluid for the duration of the experiment. Samples were centrifuged to determine if red blood cells were present; only data from rats that produced blood-free samples were analyzed. Using spectrophotometric measurement (absorbance at 620 nm), samples were then evaluated for Evans blue dye concentration which is linearly related to protein concentration (Carr and Wilhelm, 1964). Typical BK- and PAF-induced plasma extravasation responses produced by these procedures are depicted in Figure 4.

Inclusion criteria

For experiments in which a plasma extravasation response was established prior to addition of treatments (experiments from chapter 5), data were chosen for inclusion in analyses based on the following criteria:

1.) Baseline plasma extravasation levels were not greater than 0.040.

2.) Evidence that a plasma extravasation response was present as indicated by a response greater than or equal to 0.060 in at least two of the samples 5-8.

For experiments in which animals were pre-treated (experiments described in chapters 3 and 4), data were chosen for inclusion in analyses using only criterion 1 (above). Inclusion criteria are necessary because in a small percentage of animals difficulties occur which make the data uninterpretable. The requirement for low baseline virtually eliminates the possibility of contaminated vehicle since bacterial contamination would induce a plasma extravasation response. Additionally, baseline responses within a narrow range argue against the possibility of an ongoing inflammatory response. If rats were sick, it is likely that plasma extravasation in response to an exogenous inflammatory mediator would be non-specifically affected. Finally, since the occurrence of high baseline is low and it is unclear what induces it when it occurs, it seems prudent to exclude these data to avoid difficulties in interpretation. It is important to note that the incidence of high baseline is similar in animals that have been pre-treated and in animals that have not, strongly suggesting

that the pre-treatments used in this research do not themselves induce high baseline responses.

In a small percentage of cases there appears to be no observable plasma extravasation response. The reason for this is unknown but could be due to poor needle placement or degradation of the inflammatory mediators. For example, BK appears to lose activity after roughly 6 weeks at –20°C. The requirement for a discernible plasma extravasation response for some experiments is critical since the questions being posed in these experiments pertain to modulation of that response. Obviously these questions cannot be answered if a response is not observable. For experiments in which animals are pre-treated, the incidence of non-responding knees becomes a large issue. In many of the pre-treatment experiments in this series of studies the pre-treatment induces inhibition of plasma extravasation which raises the question that some knees that appear to show inhibition may actually be non-responding knees. To address this serious technical issue the following conventions have been adopted:

1.) In home cage control animals when a knee is a non-responder the plasma extravasation response is completely flat. In other words, there is no evidence whatsoever that a response is present. Therefore in animals that have been pre-treated if the response appears completely flat, those data are excluded from analysis.

2.) For these pre-treatment experiments, daily controls are used. If there is no response in daily control knees, data from the experimental knees are also excluded

based on the assumption that the lack of response in the control knees is due to degradation of the inflammatory mediator.

3.) For these experiments, large sample sizes are used to minimize any remaining effects of non-responding knees.

Stress paradigms

All stressors were administered between 8am and 11am when the circadian level of corticosterone is low in order to maximize the difference in HPA axis activation between stressed animals and home cage controls.

Ether

A box containing many openings was filled with cotton gauze. The gauze was saturated with diethyl ether and placed in the bottom of a plastic chamber. A rat was placed inside the plastic chamber, the lid was closed and the rat remained in the chamber until ten seconds after he became anesthetized (approximately five minutes total exposure). This duration of exposure was used instead of a fixed time because rats tended to require more ether to become anesthetized as the number of exposures increased. Additionally, some rats required very little ether to become anesthetized and exposure for a fixed time would have caused death. The rat was then removed from the chamber and remained in his home cage for 20 minutes. All rats were fully awake at this time point. For experiments in which a one-hour post stress time point was measured the rat was then anesthetized with (65 mg/kg, intraperitoneal) and the plasma extravasation experiment proceeded as described above. For experiments in

which a twenty-four hour post stress time point was measured, 20 minutes after removal from the ether chamber, rats were returned to the animal care facility where they remained until the following day when they were retrieved. The plasma extravasation measurement began 24 hours after the last ether exposure.

Restraint stress

Restraint stress was performed as previously described (Akana et al., 1992). Rats were placed in acrylic tubes (inner diameter: 5.7cm; length: 20.3cm) secured with tape at both ends. Once inside the tubes, rats were placed on a table in a horizontal orientation and remained there for 30 minutes. The tubes completely restricted the movement of the rats from side-to-side but they were capable of limited movement in a front-to-back direction. For experiments in which a one hour post stress time point was measured the rats were anesthetized (65 mg/kg, intraperitoneal) immediately after removal from the restraint tubes and the plasma extravasation experiment proceeded as described above. For experiments in which a twenty-four hour post stress time point was measured, rats were removed from the restraint tubes and then returned to the animal care facility until the following day when they were retrieved. The plasma extravasation measurement began 24 hours after the last restraint exposure.

Noise stress

Noise stress was performed as previously described with some minor modifications (Singh et al., 1990). Animals, in cage pairs, were placed in a 22 x 22 x

28 -inch Comatex box in a cage 10 inches away from a speaker. The box was closed and animals were exposed to a 105db noise of mixed frequencies ranging from 11KHz to 19KHz. A five- or ten-second tone was presented every minute at random intervals during the minute. The choice between a five or ten second tone was also random. This noise exposure continued for 30 minutes. For experiments in which a one hour post stress time point was measured the rats were anesthetized (65 mg/kg, intraperitoneal) immediately after removal from the noise box and the plasma extravasation experiment proceeded as described above. For experiments in which a twenty-four hour post stress time point was measured, rats were removed from the noise box and returned to the animal care facility where they remained until the following day when they were retrieved. The plasma extravasation measurement began 24 hours after their last exposure to noise. For chronic, intermittent noise exposure, the paradigm described by Singh et al. (Singh et al., 1990) was used. Specifically, rats were exposed to noise on days one, three and four of a four-day paradigm. On day two they remained in their home cages in the animal care facility.

Surgeries

Adrenalectomy

Animals were anesthetized with sodium pentobarbital (65 mg/kg). An incision was then made in the dorsal abdominal wall and the adrenal glands were exposed and removed. Following surgery and throughout the recovery and experimental periods, rats received drinking water (*ad libitum*) containing 0.5% saline and 25 μg/ml corticosterone (Akana et al., 1985). This dose of exogenous corticosterone produces

low (i.e., 8 μg/dl peak circadian value) but still phasic plasma levels of plasma corticosterone and inhibits both the enhanced ACTH secretion and decreased body weight gain observed in adrenalectomized rats without corticosterone replacement (Akana et al., 1985). Corticosterone drinking water was replaced with normal tap water 2 hours prior to the beginning of experiments to avoid any effects of the exogenous corticosterone in the drinking water. Adrenalectomy was performed 1 week prior to restraint stress experiments. Successful surgery was confirmed by measuring corticosterone levels in blood samples collected from anesthetized animals prior to sacrifice. Animals were excluded from analysis if plasma corticosterone levels were greater than 1 μg/dl.

Adrenal denervation

Innervation of the adrenal medullae was ablated as previously described (Celler and Schramm, 1981; Miao et al., 1993). In brief, animals were anesthetized with sodium pentobarbital (65 mg/kg) and an incision was made in the lateral abdominal wall. The greater splanchnic nerve innervating each adrenal gland was exposed and the "adrenal innervation region" was isolated close to the adrenal gland and cut. Adrenal denervation was performed one week prior to experiments. Since it is conceivable that the adrenal cortex could be injured during this surgical procedure, adrenal cortical function was assessed by measuring plasma corticosterone levels in stressed animals. If plasma corticosterone levels were not similar in stressed, intact and stressed, adrenal-medullae-denervated rats the rat was excluded from analysis.

Analysis of plasma corticosterone

Blood (approximately 100 µl) was collected for corticosterone measurement from the tail vein of anesthetized rats with an intravenous infusion set approximately ten minutes after administration of anesthesia. Blood samples were immediately spun in a table top centrifuge and plasma was collected. Samples were stored at -20° C until assayed. Corticosterone was measured using a radioimmune assay kit as previously described (Akana et al., 1992).

Electrical stimulation

Two stainless steel electrodes were placed transversely through the skin in the plantar area of the hindpaw 10 mm apart. Approximately 20 min after initiation of BK perfusion of the knee joint, rats received non-noxious (2.5 mA, 0.25 msec duration pulses, 3 Hz) or noxious (25 mA, 0.25 msec duration pulses, 3 Hz) electrical stimulation via the two stimulating electrodes which continued for the duration of the experiment. Stimulus intensities necessary to excite only large-diameter afferents, A-fibers, (non-noxious stimulation) and intensities that also excite small-diameter afferents, C-fibers, (noxious stimulation), were determined by electrophysiological recordings from bundles isolated from the sciatic nerve of pentobarbital anesthetized rats (Green et al., 1995).

Histology

At the end of the knee joint perfusion experiments rats were immediately perfused with 4% paraformaldehyde in PBS. The anterior fat pad of each knee joint

was then excised and post-fixed in 4% paraformaldehyde and 2% dimethyl sulfoxide. The tissue was then dehydrated and embedded in plastic. Tissue sections (6 μm thick), were stained with a modified Wright-Giemsa stain (Leukostat). Neutrophils were counted, by an investigator blinded to the identity of the sample, in approximately 6 sections, 30 μm apart, from each knee joint and an average per unit area was calculated.

Flow cytometry

Venous blood samples (500 ul) were withdrawn from rats 5 min prior to, and 30 min after, electrical stimulation. Heparin (50 units/ml) was used as an anticoagulant. Samples remained on ice until the end of the perfusion experiment when red blood cells were lysed and the remaining white cells were diluted in trypan blue and counted with a hemocytometer. Cells were always > 95% viable. Cells were stained with FITC-conjugated antibody against rat neutrophils (clone R2-1A6a) and isotype-matched FITC-conjugated antibody (clone MOPC-104E) was used as a control for non-specific staining. In separate samples, indirect immunofluorescence was used to assess L-selectin expression (clone HRL-3) and staining with FITC-conjugated secondary antibody alone served as the background control. After staining, cells were fixed (0.5 % paraformaldehyde) and assayed for fluorescence intensity with a FACScan flowcytometer. For every time point, neutrophils were gated by expression of the neutrophil marker. Cells in each gate were then analyzed in subsequent samples for expression of L-selectin. Positive L-selectin staining was

expressed as the amount of staining with both primary and secondary antibodies minus staining with the secondary antibody alone.

Laminar flow assay

Approximately 30 min after the onset of electrical stimulation, 3.5 ml blood was withdrawn via cardiac puncture using heparin (50 units/ml) as an anticoagulant. Neutrophils were isolated over discontinuous (65% / 74%) Percoll gradients (Issekutz et al., 1994). In aliquots of this neutrophil sample, remaining red blood cells were lysed, neutrophils were diluted in trypan blue and counted with a hemocytometer. Lysis and counting were repeated at least 5 times with separate aliquots. Cells were always > 95% viable. Adhesion substrates were generated by coating 4 μg/ml of peripheral node addressin (PNAd) in Tris-buffered saline (pH 8.5) onto bacteriological Petri dishes at 4 °C overnight. PNAd was obtained for use in this study by purification on a MECA-79 Sepharose column from detergent lysates of human tonsil (Berg et al., 1991). After washing with PBS, non-specific protein binding to the substrate was blocked with 3% BSA. The substrate-coated slides were incorporated as the lower wall of a parallel plate flow chamber and mounted on the stage of an inverted phase-contrast microscope. Neutrophils were perfused through the flow chamber at 0.4-2x10^6 cells/ml in HBSS with Ca^{++} and 0.1% BSA. After a 4-min baseline flow rate of 1 dyne/cm^2, flow was increased 1.5- to 2-fold, in steps, every 10 sec up to a maximum of 35 dynes/cm^2. NIH Image 1.61 was used to quantify the numbers and velocities of cells remaining rolling at each time interval.

Drug administration

Metyrapone

Metyrapone (100 mg/kg, dissolved in DMSO), a corticosterone synthesis inhibitor, was administered intraperitoneally 5 minutes prior to restraint stress for 6 days. On the seventh day, no drug was administered prior to exposure to stress. Control rats received intraperitoneal injections of DMSO on the same schedule as metyrapone injections.

ACTHar®

A single injection of ACTHar® (4 units/kg dissolved in normal saline), a slow-release formulation of ACTH, was administered subcutaneously daily for seven days between 8am and 11am. Control rats received subcutaneous injections of saline on the same schedule as ACTHar® injections.

Neutrophil depleting serum

100 µl of neutrophil-depleting serum (Johnston et al., 1996) were administered intravenously immediately before perfusion of the synovium. Control animals received an intravenous injection of pre-immune rabbit serum (100 µl) at the same time point.

Anti-L-selectin antibody

Rats received an intravenous injection of hamster anti-L-selectin monoclonal antibody (HRL-3, $F(ab)'_2$ fragments, IgG isotype; 0.5 mg/kg; total volume of 300 µl)

20-25 minutes after administration of BK to the synovium. Control rats received an intravenous injection of non-specific hamster IgG ($F(ab)'_2$ fragments; 0.5 mg/kg; total volume of 300 µl) at the same time point.

Ro31-9790

Ro31-9790, a TACE inhibitor, was wet-milled over night in Gelofusine. Ro31-9790 was administered intraperitoneally (100mg/kg in a volume of 0.5 ml) immediately before perfusion of BK through the knee joint.

Statistics

All plasma extravasation data were analyzed using repeated measures analysis of variance. Analyses of corticosterone levels and of neutrophil numbers in tissue sections were performed using standard analysis of variance. Fisher's least squares difference test (Fisher, 1949) was used for all post-hoc comparisons. The correlation analysis between plasma corticosterone and BK-induced plasma extravasation was performed by computing a Pearson product-moment correlation coefficient between plasma corticosterone and BK-induced plasma extravasation. To perform this analysis, an average plasma extravasation value was computed for each rat by averaging plasma extravasation at all time points after administration of BK. Student's t-test was used to analyze L-selectin expression and rolling chamber data.

Figure 3. Schematic diagram of knee joint model of plasma extravasation.

Fluid containing BK or PAF is perfused into the synovial space via the inflow needle and out via the outflow needle at a constant rate (250 μl/min). Perfusate samples are collected and evaluated for Evans blue dye concentration using spectrophotometric measurement.

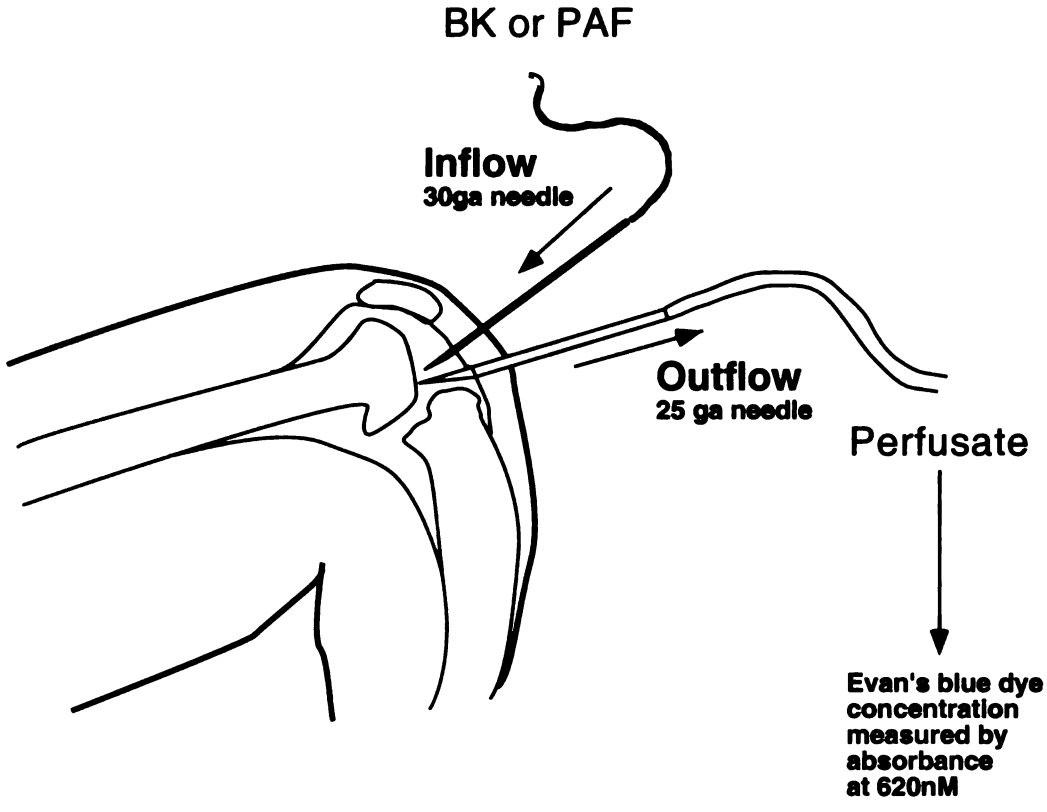
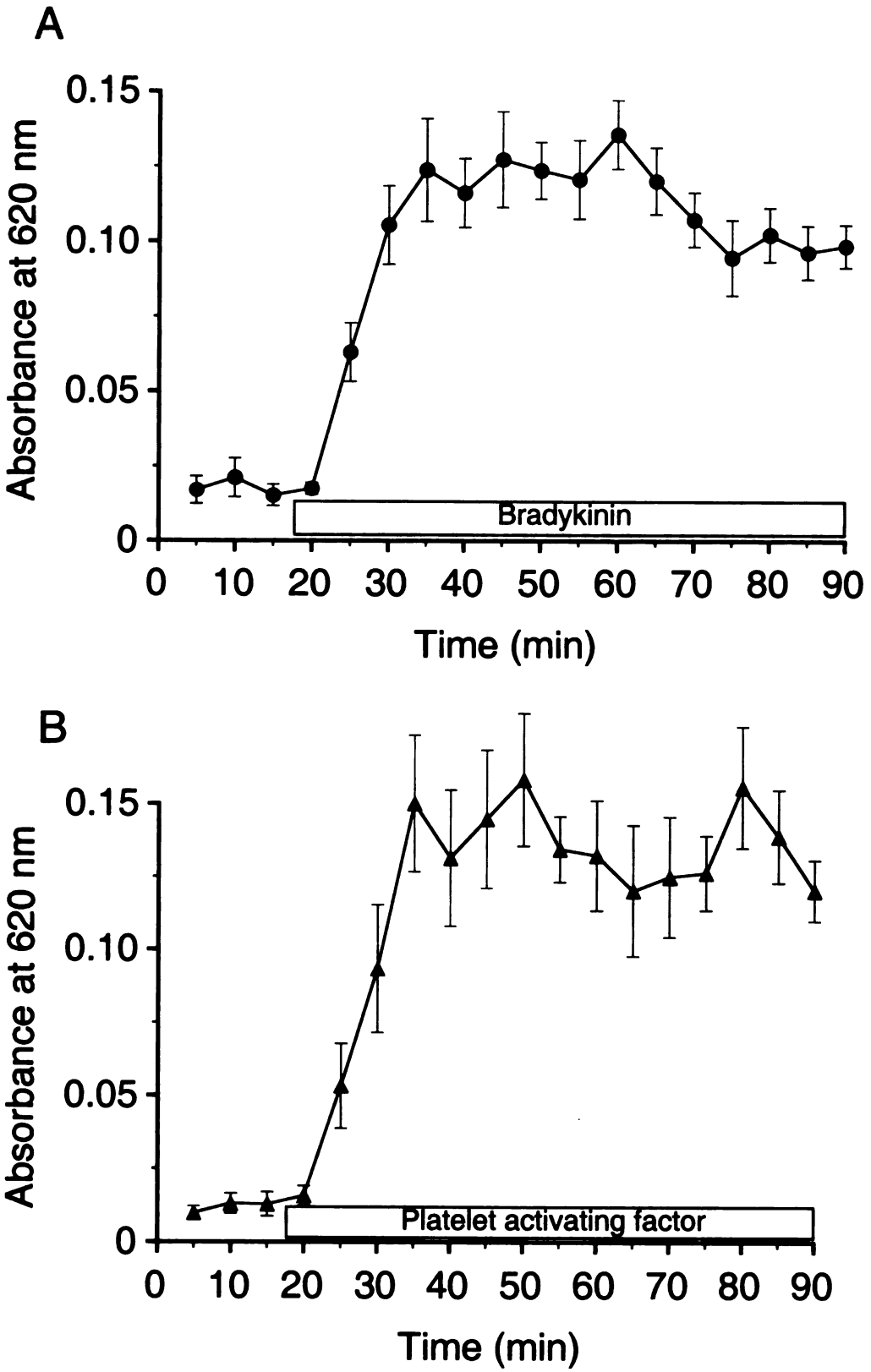

BK or PAF
Inflow
30ga needle
Outflow
25 ga needle
Perfusate
Evan's blue dye concentration measured by absorbance at 620nM

Figure 4. Typical plasma extravasation responses elicited by BK and PAF.

A. After establishing baseline levels of plasma extravasation in the first three samples, BK (150 nM) was added to the perfusing fluid (normal saline) and remained present in the fluid for the duration of the experiment. Samples were collected every 5 minutes for a period of 90 minutes.

B. After establishing baseline levels of plasma extravasation in the first three samples, PAF (35 nM) was added to the perfusing fluid (0.2% bovine serum albumin in normal saline) and remained present in the fluid for the duration of the experiment. Samples were collected every 5 minutes for a period of 90 minutes.


A
Absorbance at 620 nm
0.15
0.10
0.05
0
Bradykinin
0 10 20 30 40 50 60 70 80 90
Time (min)
B
Absorbance at 620 nm
0.15
0.10
0.05
0
Platelet activating factor
0 10 20 30 40 50 60 70 80 90
Time (min)

CHAPTER 3

CHRONIC, BUT NOT ACUTE, STRESS INHIBITS BRADYKININ-INDUCED PLASMA EXTRAVASATION

Introduction

Very little is known about the effects of stress on the inflammatory response. What is known is drawn from human studies that have correlated self-reports of stressful life events and exacerbation of signs and symptoms of chronic inflammatory diseases as well as a few animal studies that have investigated the effects of stress on defined components of the inflammatory response. The human studies have in many cases established positive correlations between exacerbation of inflammatory diseases and psychological stress. For example, mild daily stressors exacerbate symptoms of rheumatoid arthritis (Potter and Zautra, 1997; Zautra et al., 1994), ulcerative colitis (Levenstein et al., 1994) and inflammatory bowel disease (Dancey et al., 1998). Additionally, higher levels of perceived stress are associated with increased incidence of asthmatic episodes (Sekas and Wile, 1980). Both patients with rheumatoid arthritis (Affleck et al., 1987) and irritable bowel syndrome (Robertson et al., 1989) cite stress as the most frequent cause of symptom flares and stress management interventions can reduce these symptom flares (Parker et al., 1995). Little is known about the nature of the stressors in these studies since it is difficult to isolate specific stressors and their characteristics when the experiments use naturally occurring stressors in the subjects' daily lives. However, in one rare study that did attempt to investigate the nature of the stressors involved Potter et al. (Potter and Zautra, 1997) found that mild daily life stress is highly correlated with symptom flares in inflammatory disease while major life stresses are actually negatively correlated with flares.

Information from animal studies that investigate the effects of stress on inflammation is based on three studies. Acute stress decreases the plasma

extravasation response to inflammatory stimuli (Bhattacharya et al., 1987; Harmsen and Turney, 1985). Additionally, the only study to investigate the effects of stress on neutrophil function demonstrated that stress inhibits inflammatory stimulus-induced neutrophil accumulation *in vivo* but increases neutrophil adherence to plastic *in vitro* (Harmsen and Turney, 1985). Only one study has investigated the effects of chronic stress on any inflammatory component. Specifically, Padgett and co-workers (Padgett et al., 1998) have shown that chronic restraint stress decreases the amount of cellular infiltrate 1 and 3 days after initiation of the wound in a model of cutaneous wound healing. No reported studies have investigated the effects of stress on the inflammatory response continuously over a substantial time period. Additionally, there is very little information available pertaining to the mechanisms of the reported stress-induced effects.

In view of the limited information available pertaining to the effects of stress on inflammation, the present inquiry begins by investigating very basic questions. The following experiments are designed to ask first whether stress affects inflammation at all in the rat knee joint model. Second, since acute and chronic stressors have been shown to have disparate effects on other immune responses (Batuman et al., 1990; Irwin and Hauger, 1988) these experiments will also determine whether acute and chronic stress affect inflammation differently. Third, to begin to understand the potential mechanisms of the effects of stress on inflammation, its effects on both neurogenic and non-neurogenic inflammation will be explored. Since neurogenic (BK-induced) and non-neurogenic (PAF-induced) inflammation involve participation of different cellular components (Green et al., 1993), differential effects

of stress on these two types of inflammation may yield information about possible targets of stress effects. Finally, to determine the generalizability of the stress effects, several stressors will be investigated.

Results

Ether is a stressful stimulus.

Similar to findings of others (Cook et al., 1973; Gaillet et al., 1991; Wilkinson et al., 1981), ether stress induced a large increase in plasma corticosterone (Figure 5; $F(2,16) = 16.8$, $p < 0.001$; post-hoc 1-day ether versus home-cage, anesthetized control, $p < 0.001$). After seven daily exposures, ether stress continued to elicit a robust plasma corticosterone response that was not significantly different than the response to one exposure to ether stress indicating that the adrenal did not habituate to this stressor (Figure 5; $F(2, 16) = 16.8$, $p < 0.001$; post-hoc 1-day ether versus 7-day ether, ns; 7-day ether versus home cage control, $p < 0.001$). As an independent confirmation that the ether stimulus was perceived as stressful throughout the seven-day exposure period, we measured rat body weights. Rats exposed to daily ether stress gained significantly less body weight than control rats exposed to similar housing conditions (Figure 6; $F(1,8) = 71.5$, $p < 0.001$).

Chronic intermittent ether stress inhibits BK-induced plasma extravasation 24 hours after the seventh daily exposure.

To determine the effects of acute ether stress on BK-induced plasma extravasation, we exposed rats to ether stress and measured BK-induced plasma

extravasation either one or twenty-four hours after the stress exposure. BK-induced plasma extravasation was not significantly different between animals that had been exposed to acute ether stress and home cage controls at either time point indicating that acute ether stress does not affect BK-induced plasma extravasation (Figure 7; $F(2, 21) = 0.29$, ns).

To determine the effects of chronic, intermittent ether stress on BK-induced plasma extravasation, we exposed rats to daily ether stress for seven days and measured BK-induced plasma extravasation either one or twenty-four hours after the last stress exposure. When measured one hour after the last stress exposure, BK-induced plasma extravasation was not significantly different between animals that had been exposed to chronic, intermittent ether stress and home cage controls indicating that chronic ether stress does not affect BK-induced plasma extravasation at that time point (Figure 8; $F(2,23) = 10.7$, $p < 0.001$; post-hoc 7-day ether + 1 hour versus home-cage control, ns). However, when measured twenty-four hours after the seventh ether exposure BK-induced plasma extravasation is profoundly inhibited compared to home cage controls (Figure 8; $F(2,23) = 10.7$, $p < 0.001$; post-hoc 7-day ether + 24 hours versus home-cage control or 7-day ether + 1 hour, $p < 0.001$).

Restraint is a stressful stimulus.

To determine if the effects observed with ether stress are generalizable to other stressors, we tested the effects of restraint stress on plasma extravasation. Restraint is widely used in the literature as a stressful stimulus (Akana et al., 1992; Bhatnagar et al., 1995; Gaillet et al., 1991; Padgett et al., 1998) and has the advantage that it is non-

invasive and is therefore unlikely to have non-specific effects on inflammation. Restraint stress induced a large increase in plasma corticosterone (Figure 9; $F(2,22) = 10.0$, $p < 0.001$; post-hoc 1-day restraint versus home-cage, anesthetized control, $p < 0.001$). Similar to findings of others (De Boer et al., 1990; Dhabhar et al., 1997), the adrenal shows habituation of this response and after seven daily exposures to restraint stress the evoked plasma corticosterone response is significantly less than the response to one exposure (Figure 9; $F(2,22) = 10.0$, $p < 0.001$; post-hoc 1-day restraint versus 7-day restraint, $p < 0.05$) but still induces a significant increase in plasma corticosterone when compared to home-cage controls (Figure 9; $F(2,22) = 10.0$, $p < 0.001$; post-hoc 7-day restraint versus home-cage, anesthetized control, $p < 0.05$). As an independent confirmation that the restraint stimulus was perceived as stressful throughout the seven-day exposure period, we measured rat body weights. Rats exposed to daily restraint stress gained significantly less body weight than control rats exposed to similar housing conditions (Figure 10; $F(1,12) = 7.8$, $p < 0.05$).

Chronic intermittent restraint stress inhibits BK-induced plasma extravasation one hour after stress exposure.

To determine the effects of restraint stress on BK-induced plasma extravasation, we exposed rats to 30 minutes of restraint stress and measured BK-induced plasma extravasation one and twenty-four hours later. BK-induced plasma extravasation was not significantly different between animals that had been exposed to acute restraint stress and home cage controls at either time point indicating that acute

restraint stress does not affect BK-induced plasma extravasation (Figure 11; $F(2,26) = 0.24$, ns).

To determine the effects of chronic, intermittent restraint stress on BK-induced plasma extravasation, we exposed rats to daily restraint stress for seven days and measured BK-induced plasma extravasation either one or twenty-four hours after the last stress exposure. When measured one hour after the last stress exposure, BK-induced plasma extravasation was significantly inhibited when compared to home cage controls (Figure 12; $F(2, 28) = 17.8$, $p < 0.001$; post-hoc 7-day restraint + 1 hour versus home-cage, anesthetized control, $p < 0.001$). Unlike ether stress, twenty-four hours after chronic restraint stress exposure there was no significant difference in BK-induced plasma extravasation between rats exposed to restraint stress and home cage controls suggesting that these stressors may have different mechanisms of effect (Figure 12, $(2, 28) = 17.8$, $p < 0.001$; post-hoc 7-day restraint + 24 hours versus home-cage, anesthetized control, ns).

Noise is a stressful stimulus.

The different latencies of the effects of restraint and ether stress suggest that these two stressors may differ in their mechanisms of action. It must be noted, however, that ether is a pharmacological agent with pleiotropic effects, some of which could have direct effects on plasma extravasation. For example, ether is a potent vasodilator and could induce increases in blood flow that might non-specifically increase plasma extravasation. To determine if the difference in effect latencies

between ether and restraint stress are due to stress effects or non-specific effects of ether, we investigated the effects of a third stressor on plasma extravasation.

Noise stress has been used by other investigators (Feldman et al., 1972; Lau, 1992; Singh et al., 1990) and induces a robust activation of the HPA axis as measured by plasma corticosterone (Feldman et al., 1972; Lau, 1992). Moreover, noise stress is non-invasive and is therefore unlikely to have non-specific effects on inflammation. Noise stress induced a large increase in plasma corticosterone (Figure 13; $F(2, 20) = 12.4$, $p < 0.001$, post-hoc 1-day noise versus home-cage anesthetized controls, $p < 0.001$). The adrenal did not habituate to this stressor as increases in plasma corticosterone observed after the chronic, intermittent four-day noise stress paradigm were similar to those observed after one exposure (Figure 13; $F(2, 20) = 12.4$, $p < 0.001$, post-hoc 1-day noise versus 4-day noise, ns). Additionally, rats exposed to noise stress gained significantly less body weight than control rats exposed to similar housing conditions providing an independent indication that the noise stress paradigm is stressful (Figure 14; $F(1,15) = 13.2$. $p < 0.05$).

Chronic intermittent noise stress inhibits BK-induced plasma extravasation 24 hours after the four-day paradigm.

To determine the effects of acute noise stress on BK-induced plasma extravasation, we exposed rats to noise stress and measured BK-induced plasma extravasation one and twenty-four hours after the stress exposure. BK-induced plasma extravasation was not significantly different between animals that had been exposed to acute noise stress and home cage controls at either time point indicating that acute

noise stress does not affect BK-induced plasma extravasation (Figure 15; $F(2,32) = 0.32$. ns).

To determine the effects of chronic, intermittent noise stress on BK-induced plasma extravasation, we exposed rats to the four-day noise stress paradigm described by others (Singh et al., 1990) and measured BK-induced plasma extravasation one and twenty-four hours after the last stress exposure. When measured one hour after the final stress exposure, BK-induced plasma extravasation was not significantly different between animals that had been exposed to chronic, intermittent noise stress and home cage controls indicating that chronic noise stress does not affect BK-induced plasma extravasation at that time point (Figure 16; $F(2,37) = 11.9$, $p < 0.001$; post-hoc 4-day noise + 1 hour versus home-cage, anesthetized control, ns). However, when measured twenty-four hours after the final noise exposure, BK-induced plasma extravasation was profoundly inhibited compared to home cage controls indicating that noise and ether stress exhibit a similar latency of effect on plasma extravasation (Figure 16; $F(2,37) = 11.9$, $p < 0.001$; post-hoc 4-day sound + 24 hours versus home-cage anesthetized control or 4-day noise + 1 hour, $p < 0.001$).

Chronic intermittent stress inhibits neurogenic but not non-neurogenic plasma extravasation.

Thus far the data indicate that all three stressors inhibit BK-induced plasma extravasation. However, there appear to be two types of effects with respect to latency as the effects of restraint stress are immediately apparent while those of both ether and noise are not observable until 24 hours after the final exposure to the stressor. These

differences in latency may be indicative of differences in mechanism. Therefore, to begin to understand the mechanism of these effects, we investigated the effects of each type of stressor on non-neurogenic (PAF-induced) plasma extravasation. The mechanism of PAF-induced plasma extravasation is different than that of BK-induced plasma extravasation in that it does not involve sympathetic post-ganglionic efferents or neutrophils (Green et al., 1993).

To determine the effect of chronic, intermittent restraint stress on PAF-induced plasma extravasation, we exposed rats to the seven-day restraint stress paradigm and then measured PAF–induced plasma extravasation. Unlike BK-induced plasma extravasation, PAF-induced plasma extravasation was not inhibited by chronic, intermittent restraint stress. Interestingly, rats exposed to seven daily restraint episodes exhibited a small, but significant *increase* in PAF-induced plasma extravasation when compared to home cage control rats indicating that restraint stress does not affect non-neurogenic inflammation in the same way that it affects neurogenic inflammation (Figure 17; $F(2,49) = 28.2$, $p < 0.001$; post-hoc 7-day restraint + 1 hour with PAF versus home cage anesthetized controls, $p < 0.001$).

To determine the effect of chronic, intermittent noise stress on PAF-induced plasma extravasation we exposed rats to the four-day noise stress paradigm and then measured PAF–induced plasma extravasation. Of the delayed latency category of stressors, noise stress was chosen for this further investigation instead of ether stress since ether has potential non-specific pharmacological effects. Similar to the effects of chronic restraint stress, chronic noise stress induced a small, but significant increase in PAF-induced plasma extravasation when compared to home cage controls

(Figure 18; $F(2,34) = 11.8$, $p < 0.001$; post-hoc 4-day noise + 24 hours with PAF versus home cage anesthetized controls, $p < 0.001$).

Discussion

In this study we have shown that chronic but not acute stress profoundly inhibits BK-induced plasma extravasation. Chronic stress does not inhibit non-neurogenic inflammation and in fact slightly potentiates it. These effects are likely to be generalizable since more than one type of stressor induces them. However, two different latencies of stress-induced inhibition of plasma extravasation are exhibited among the stressors suggesting that all of the stressors do not exert their effects on plasma extravasation by identical mechanisms.

The observation that chronic but not acute stress inhibits BK-induced plasma extravasation is consistent with findings in the literature that describe the effects of stress on *in vitro* immune parameters. For example, animals exposed to chronic but not to acute stress exhibit inhibited natural killer cell cytoxicity (Irwin and Hauger, 1988), decreased mitogen-induced splenocyte proliferation and decreased IL-2 production by stimulated splenic T cells (Batuman et al., 1990). The mechanism by which repeated exposures to a stressor induce different effects than a single exposure to the same stressor is unknown but there are many possibilities. For example, it is possible that plasma hormones elicited by stressful stimuli are not the direct mediators of the stress effects but rather that these hormones induce secondary factors that mediate the effects. If these factors require protein synthesis, effects would not be likely to be observed immediately after exposure to the stressors. However, in this study since

plasma extravasation was measured twenty-four hours after acute stress and effects were not present, induction of putative secondary factors would require greater than 24 hours. There is some support for this idea in the literature. Glucocorticoids are known to induce upregulation of β_2-adrenergic receptors (Bronnegard et al., 1995; Mak et al., 1995; Steinkraus et al., 1996) which play a role in the regulation of BK-induced plasma extravasation (Coderre et al., 1991). When glucocorticoids are administered *in vivo*, β_2-adrenergic receptor upregulation does not occur in response to one injection of glucocorticoids but rather repeated daily injections of glucocorticoids are required (Mak et al., 1995). It must be noted that glucocorticoid injection may induce very different results than stress, nevertheless this remains an interesting potential mechanism that merits further study.

An alternative hypothesis is that repeated exposure to stress-induced elevations in specific hormones may alter the stress axes such that subsequent homotypic stress produces effects that are not observed in naïve animals. For example, this plasticity is observed in the HPA axis in that chronically but not acutely stressed rats show decreased expression of arginine vasopressin (AVP) in the amygdaloid nuclei (Albeck et al., 1997). This decreased AVP expression is strongly correlated with decreased plasma testosterone levels (Albeck et al., 1997) and is consistent with the observation that chronic but not acute stress decreases plasma testosterone levels (Batuman et al., 1990). This may be relevant to the mechanism of stress-induced inhibition of plasma extravasation since plasma testosterone is positively correlated with level of BK-induced plasma extravasation (Green et al., in press).

A third hypothesis is that rats mount protective responses to counteract the effects of a single exposure to stress but that after repeated exposures to the stressor host protection is exhausted and the rat is no longer able to mount protective responses. Indeed as early as 1936, Selye suggested this (Selye, 1936). Specifically, he observed that animals were often able to counteract effects of acute stress but were unable to maintain this protective response and after prolonged exposure to stressors developed "diseases of adaptation." It is important to note that in those experiments "prolonged exposure" was on the order of weeks and in this study the duration of chronic stress is days. However, Selye used different stressors and measured different endpoints. So it is possible that the observed chronic stress effects are not due to induction of specific factors but instead are due to failure to maintain a protective response.

Although further study is certainly required to determine the mechanism of chronic stress induced inhibition of BK-induced plasma extravasation, the current study provides some information. In this study chronic stress inhibited neurogenic plasma extravasation and enhanced non-neurogenic plasma extravasation. Previous studies in this model have shown that neurogenic (BK-induced) and non-neurogenic plasma extravasation (PAF-induced) involve participation of different cellular components (Green et al., 1993). Specifically, neurogenic plasma extravasation requires the presence of neutrophils and sympathetic post-ganglionic nerve terminals while non-neurogenic plasma extravasation does not require participation of these cells. Since stress induces inhibition of neurogenic but not non-neurogenic plasma extravasation it is likely that the mechanism of inhibition may involve neutrophils or

sympathetic post-ganglionic nerve terminals. There is some evidence that stress can inhibit neutrophil recruitment *in vivo* (Harmsen and Turney, 1985) which provides a further suggestion that neutrophils may be a reasonable target for stress hormones to mediate the observed inhibition of neurogenic plasma extravasation. Additionally the presence of adrenergic receptors on sympathetic post-ganglionic efferent terminals and the demonstrated ability of glucocorticoids to enhance adrenergic receptor expression (Bronnegard et al., 1995; Mak et al., 1995; Steinkraus et al., 1996) further support the idea of the sympathetic post-ganglionic efferent terminal as a mediator of the observed inhibitory effects of stress. The mechanism of the observed enhancement of PAF-induced plasma extravasation by stress is an open question. It must be noted, however, that these effects are small and therefore it may be difficult to investigate this mechanism.

Interestingly, two different latencies of effects were exhibited in this study. Chronic restraint stress inhibited plasma extravasation one hour but not twenty-four hours after the final stress exposure while both ether and noise stress inhibited plasma extravasation twenty-four hours after the final stress exposure but not one hour after the final stress exposure. This difference may provide more information regarding the mechanism of stress-induced inhibition of neurogenic plasma extravasation as it may indicate that two separate mechanisms are operating and some characteristic of the stressor differentially induces each mechanism. The literature does indicate that different stressors may effect inflammation in different ways. For example, Potter et al. (Potter and Zautra, 1997) found that mild daily life stress is highly correlated with symptom flares in patients with inflammatory disease while major life stresses are

actually negatively correlated with flares. It is also known that although all stress axes are activated by any stressful stimulus, the amount of stress hormone produced by each axis can be different for different stressors (Kopin, 1995). Therefore it is possible that more than one stress hormone can elicit inhibition of plasma extravasation and that these stress hormones can induce inhibition by different mechanisms. There is evidence in this study that the stress paradigms do not affect the rats in an identical manner. Chronic stress levels of plasma corticosterone (Figures 5, 9 and 13: ether 39μg/dl +/- 5, noise 33μg/dl +/- 7 and restraint 24μg/dl +/- 7) are higher for noise and ether compared to restraint and rats exposed to ether or noise gain less body weight than rats exposed to restraint stress (ether 3.4 g +/- 1.3 g, noise 2.1 g +/- 2.1g and restraint 5.3 g +/- 1.7g). Additionally, the evoked corticosterone responses to noise and ether do not habituate after repeated exposure (Figures 5, 13) while the corticosterone response to restraint stress does (Figure 9). The overall mechanism of the stress effects on plasma extravasation as well as the potential differences in mechanism between stressors are intriguing topics that require further study. An investigation of the mechanism by which restraint stress inhibits BK-induced plasma extravasation is the subject of the next chapter.

Figure 5. Acute and chronic, intermittent ether exposure evokes increases in plasma corticosterone.

Plasma corticosterone was measured in anesthetized rats one hour after one exposure to ether (1 day, n=5) or one hour after the seventh daily exposure to ether (7 day, n=4). Plasma corticosterone was measured in anesthetized, home cage control rats (control, n=10) for comparison. Plasma corticosterone levels did not habituate after seven daily exposures to ether.

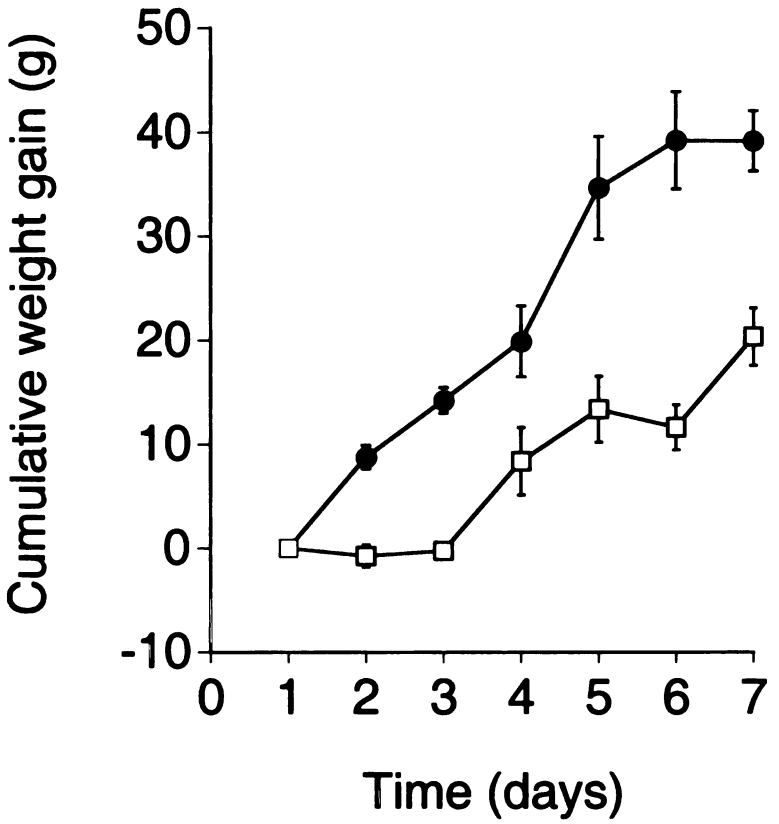

Cumulative weight gain (g)
50
40
30
20
10
0
-10
0
1
2
3
4
5
6
7
Time (days)

Figure 6. Daily ether exposure decreases body weight gain.

Rats exposed daily to ether (open squares, n=4-8 per group) gained significantly less body weight over time compared to home-cage controls (filled circles, n=7-13 per group) providing an independent verification that daily ether exposure is stressful.

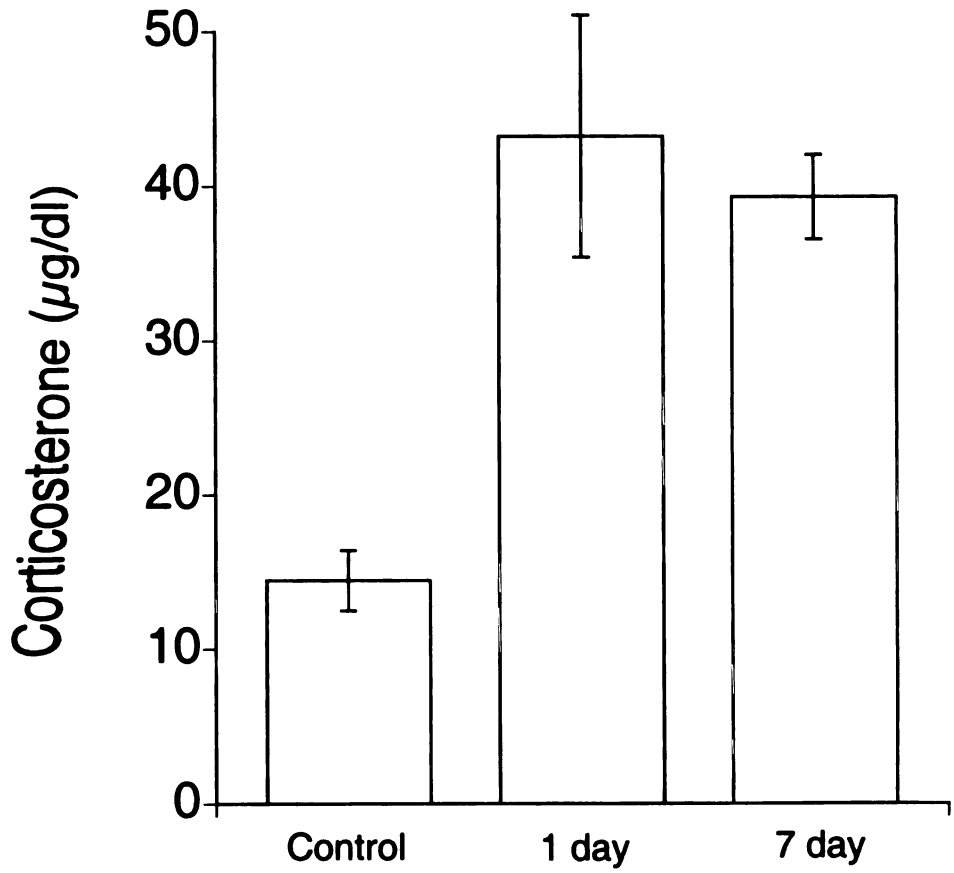

Corticosterone (μg/dl)
50
40
30
20
10
0
Control
1 day
7 day

Figure 7. Acute ether stress does not affect bradykinin-induced plasma extravasation.

BK induces sustained plasma extravasation in home cage control rats (filled circles, n=8). One exposure to ether stress does not affect BK-induced plasma extravasation whether it is measured one hour after ether exposure (open triangles, n=7) or twenty-four hours after ether exposure (open diamonds, n=9).

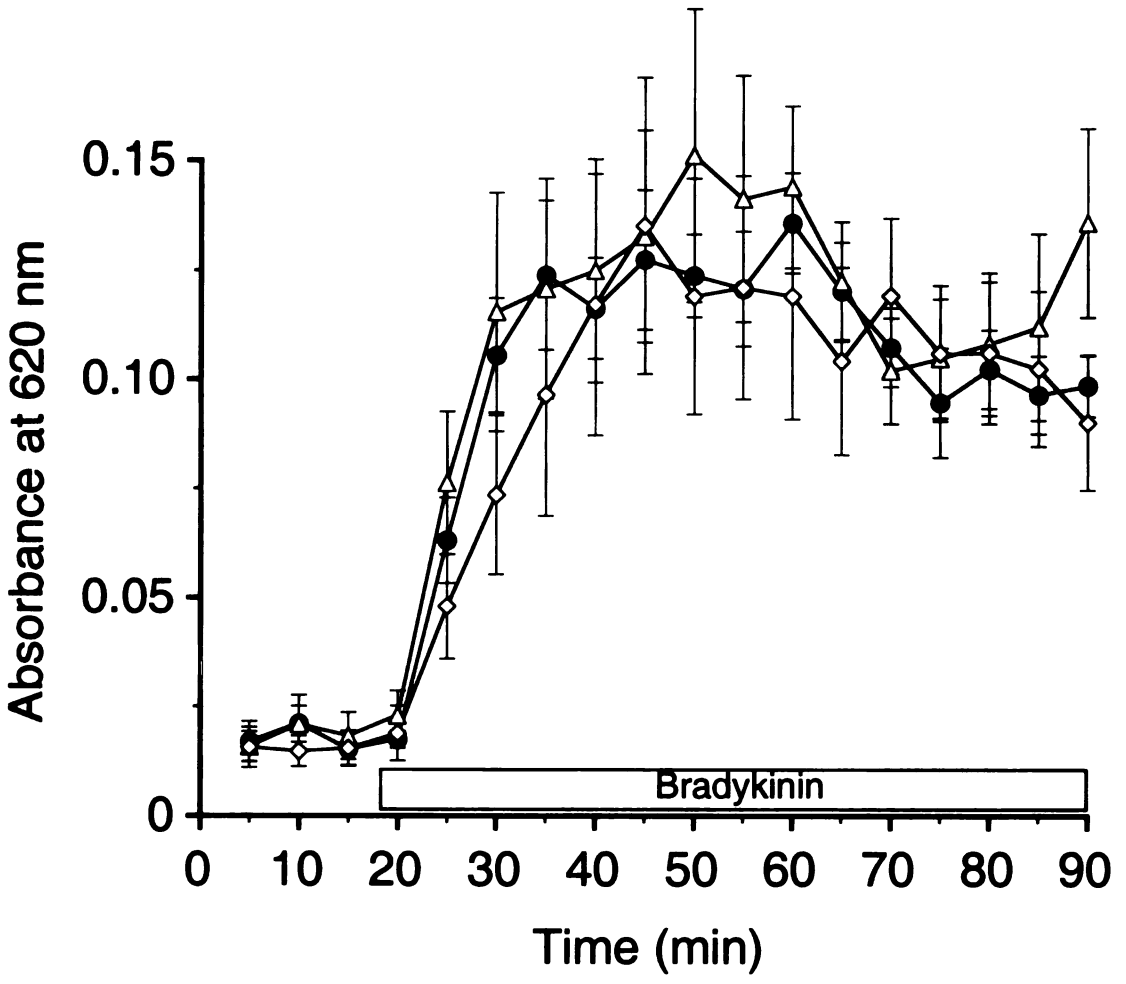
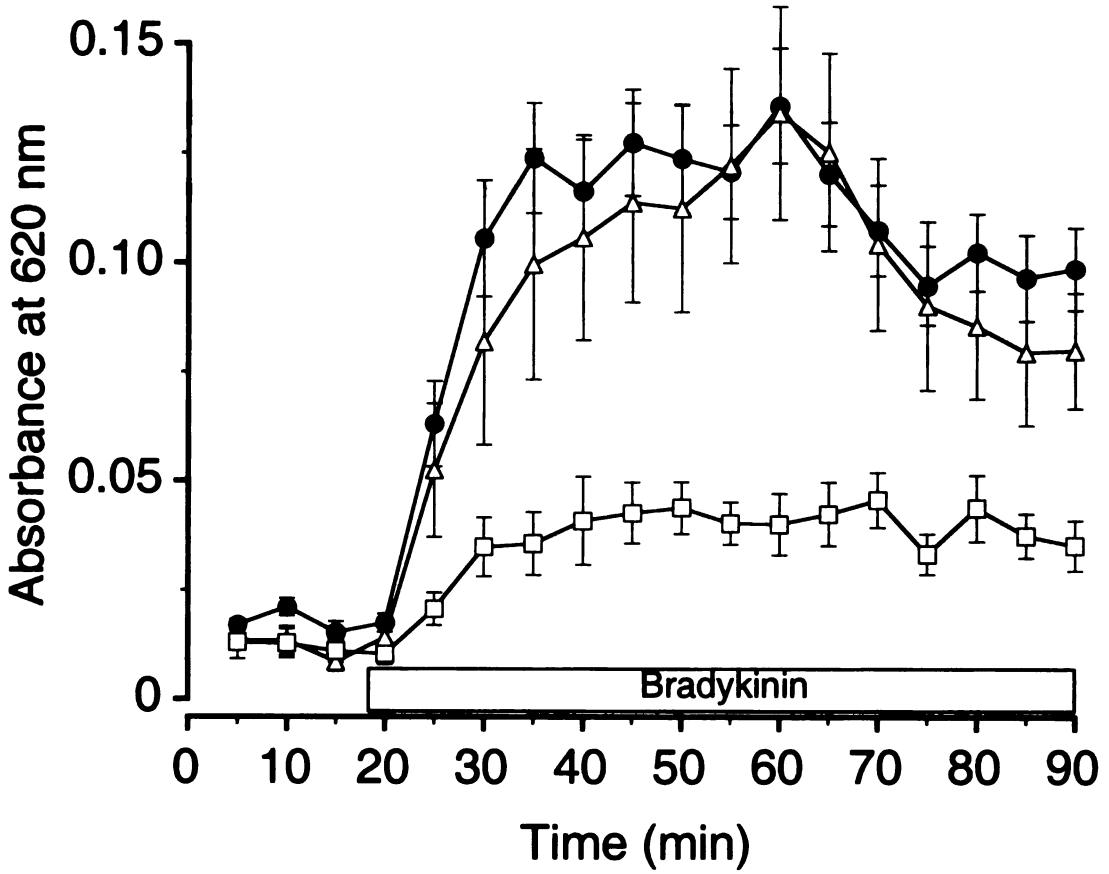

0.15
0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 8. Chronic, intermittent ether stress inhibits BK-induced plasma extravasation.

BK-induced plasma extravasation is markedly inhibited twenty-four hours after the seventh daily exposure to ether (open squares, n=9) but is unaffected when measured one hour after the seventh daily ether exposure (open triangles, n=9). A curve depicting the plasma extravasation response to BK in home-cage control animals (filled circles, n=8) is reproduced from Figure 7 for comparison.


0.15
0.10
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0
Absorbance at 620 nm
Bradykinin
0
10
20
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40
50
60
70
80
90
Time (min)

Figure 9. Acute and chronic, intermittent restraint exposure evokes increases in plasma corticosterone.

Plasma corticosterone was measured in anesthetized rats one hour after one exposure to restraint (1 day, n=8) or one hour after the seventh daily exposure to restraint (7 day, n=7). Plasma corticosterone in anesthetized, home cage control rats (control, n=10) is reproduced from Figure 5 for comparison. Plasma corticosterone levels habituate after seven daily exposures to restraint.

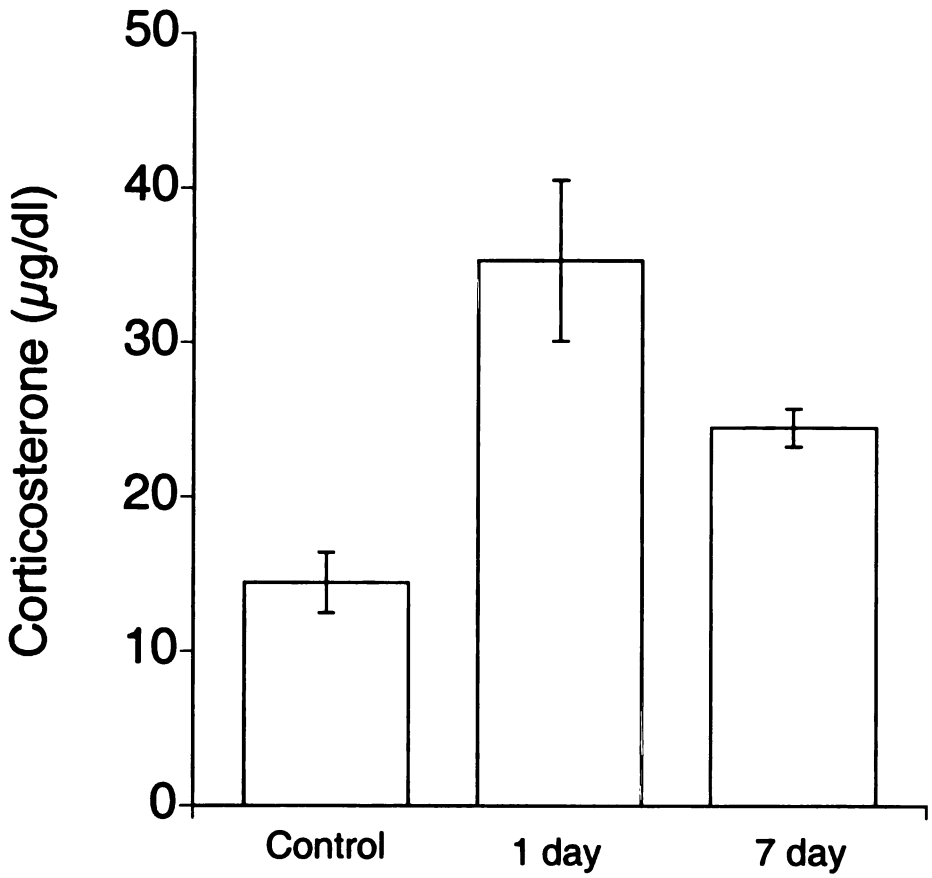

Corticosterone (μg/dl)
50
40
30
20
10
0
Control
1 day
7 day

Figure 10. Daily restraint exposure decreases body weight gain.

Rats exposed daily to restraint (open triangle, 8-17 per group) gained significantly less body weight over time compared to home-cage controls (filled circles, 7-13 per group) providing an independent verification that daily restraint exposure is stressful.

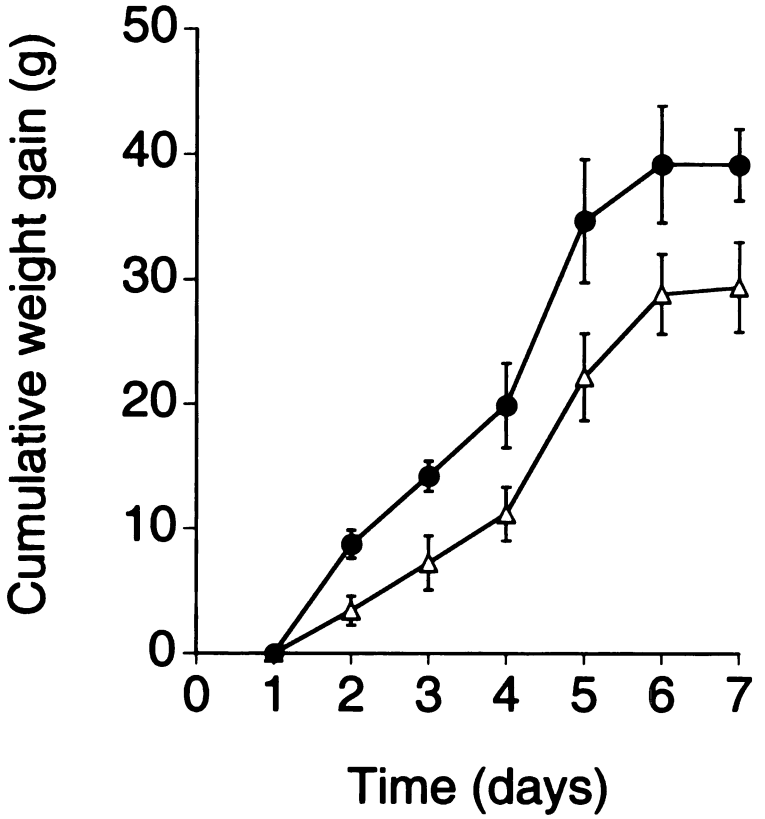

Cumulative weight gain (g)
50
40
30
20
10
0
0 1 2 3 4 5 6 7
Time (days)

Figure 11. Acute restraint stress does not affect BK-induced plasma extravasation.

BK induces sustained plasma extravasation in home cage control rats (filled circles, n=10). One exposure to restraint stress does not affect BK-induced plasma extravasation whether it is measured one hour after restraint exposure (open triangles, n=13) or twenty-four hours after restraint exposure (open diamonds, n=5).

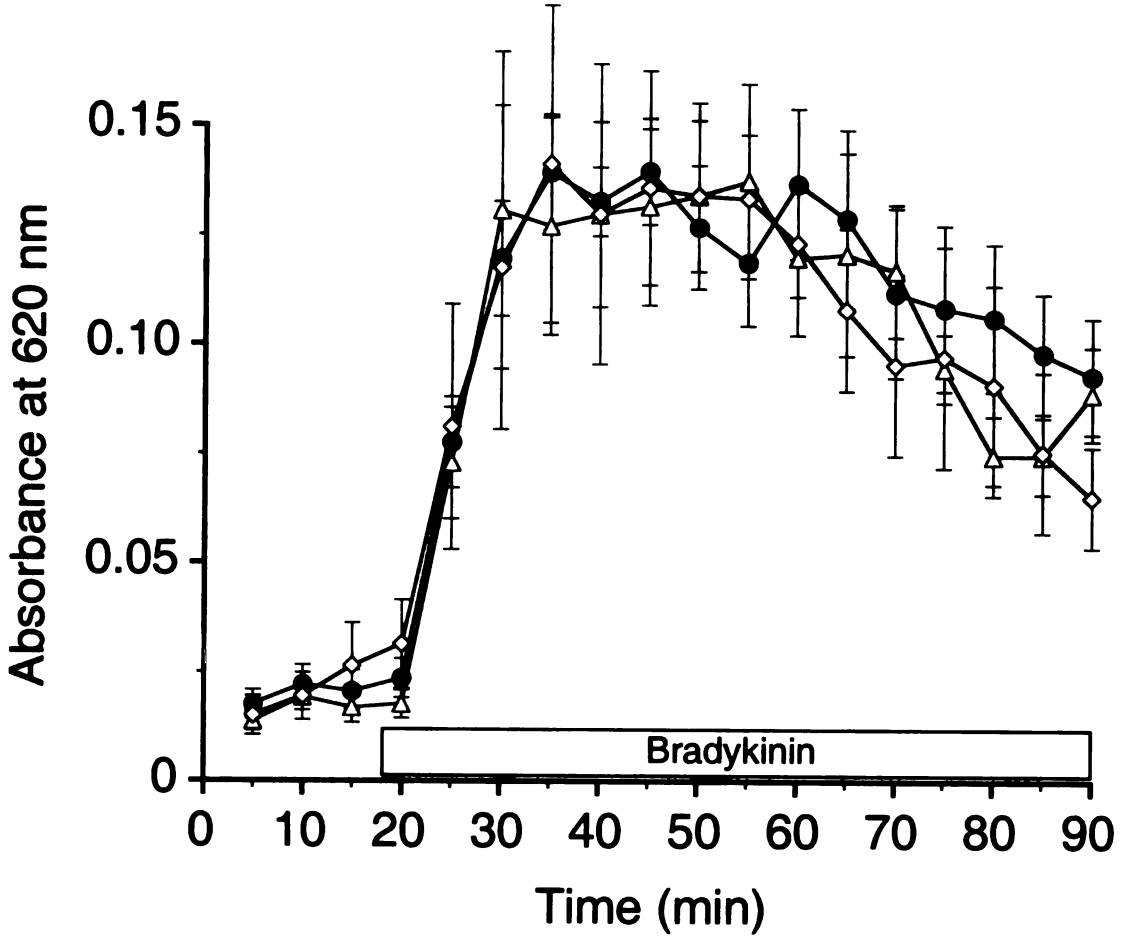
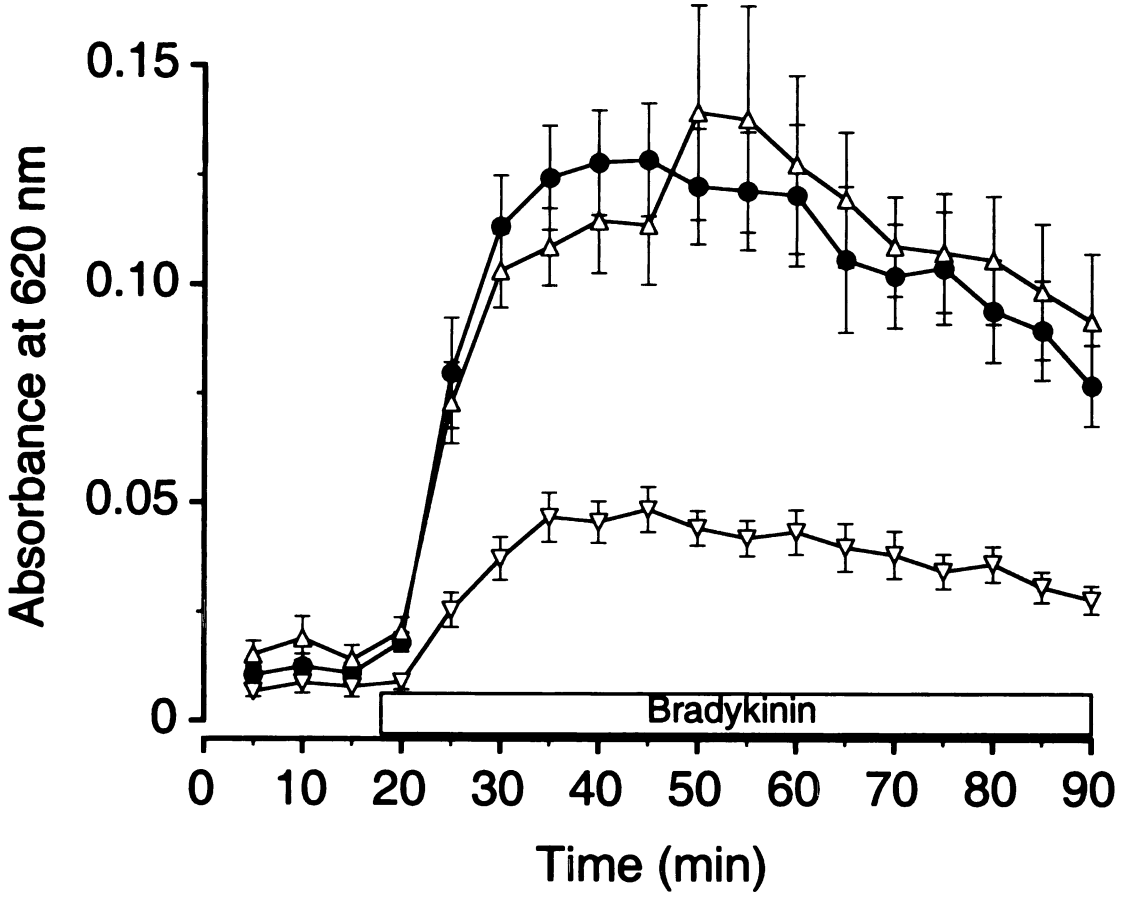

0.15
0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 12. Chronic, intermittent restraint stress inhibits BK-induced plasma extravasation.

BK-induced plasma extravasation is markedly inhibited one hour after the seventh daily exposure to restraint (open, inverted triangles, n=10) but returns to control levels twenty-four hours after the seventh daily restraint exposure (open triangles, n=7). A curve depicting the plasma extravasation response to BK in home-cage control animals (filled circles, n=14) is included for comparison.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0.10
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0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 13. Acute and chronic, intermittent noise exposure evokes increases in plasma corticosterone.

Plasma corticosterone was measured in anesthetized rats one hour after one exposure to noise (1 day, n=7) or one hour after the seventh daily exposure to noise (7 day, n=6). Plasma corticosterone in anesthetized, home cage control rats (control, n=10) is reproduced from Figure 5 for comparison. Plasma corticosterone levels did not habituate after seven daily exposures to ether.

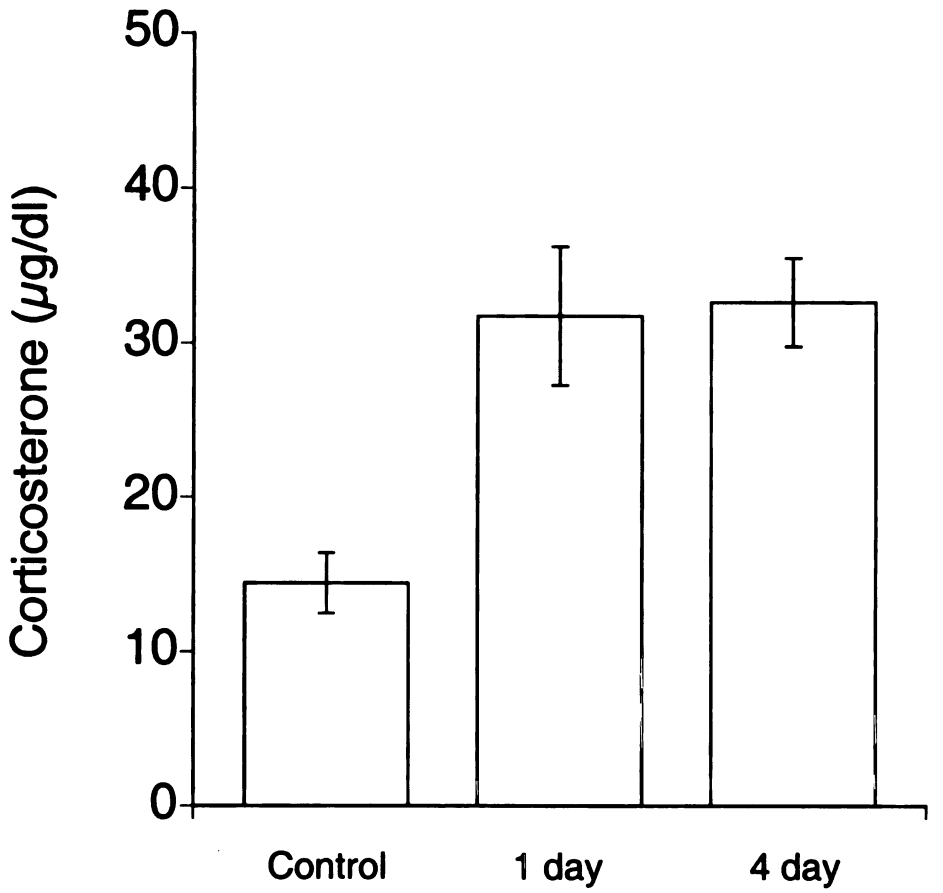

Corticosterone (µg/dl)
50
40
30
20
10
0
Control
1 day
4 day

Figure 14. Daily noise exposure decreases body weight gain.

Rats exposed daily to noise (open diamonds, n=10 per group) gained significantly less body weight over time compared to home-cage controls (filled circles, n=7-13 per group) providing an independent verification that daily noise exposure is stressful.

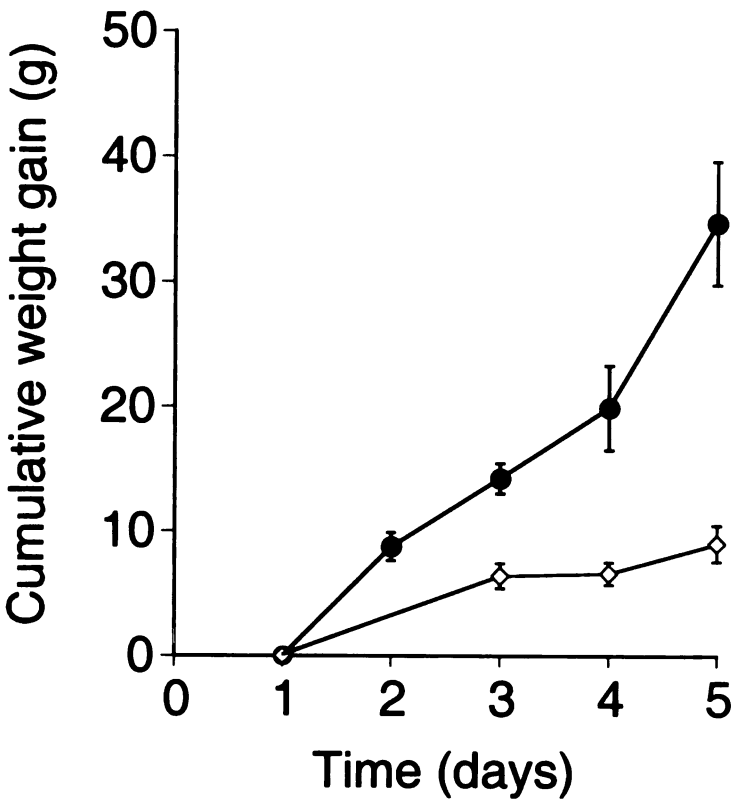

Cumulative weight gain (g)
50
40
30
20
10
0
0
1
2
3
4
5
Time (days)

Figure 15. Acute noise stress does not affect BK-induced plasma extravasation.

One exposure to noise stress does not affect BK-induced plasma extravasation whether it is measured one hour after noise exposure (open triangles, n=8) or twenty-four hours after noise exposure (open diamonds, n=9). A curve depicting the plasma extravasation response to BK in home-cage control animals (filled circles, n=18) is included for comparison.

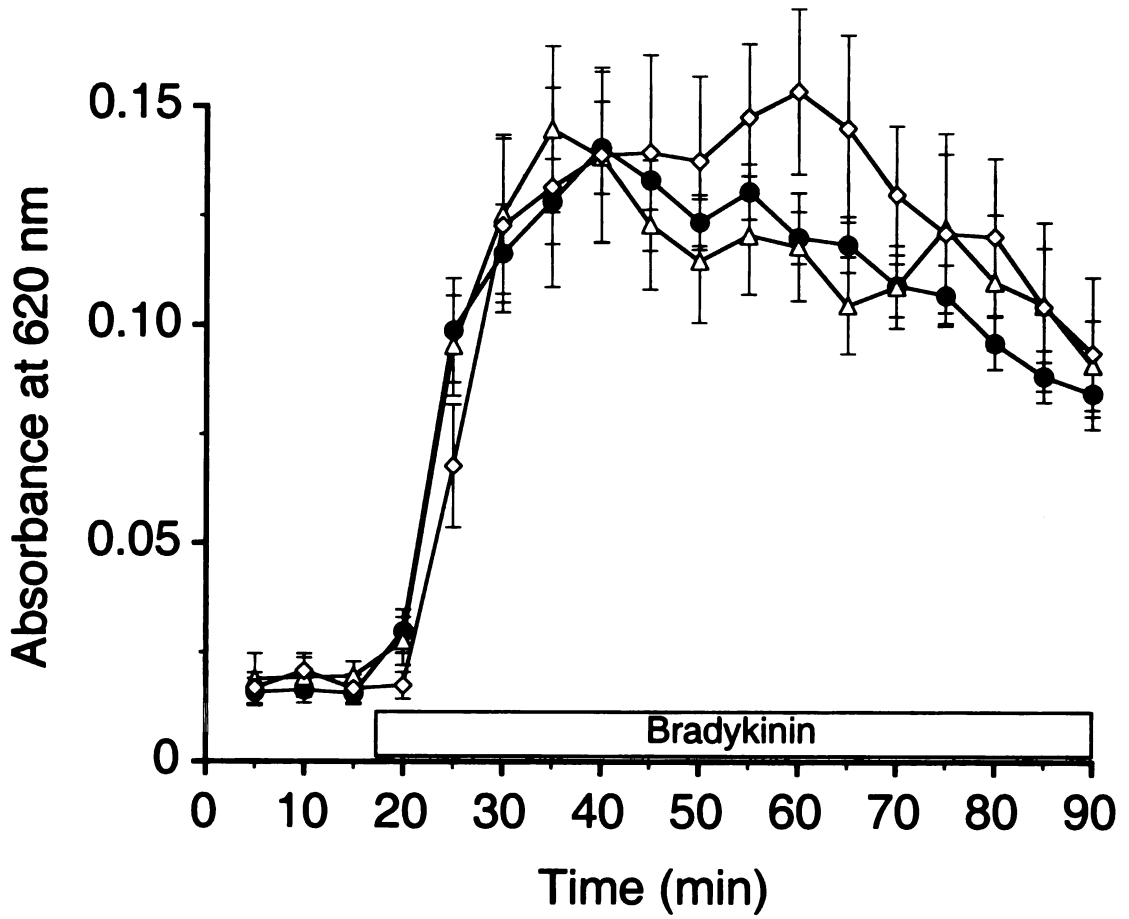

0.15
0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 16. Chronic, intermittent noise stress inhibits BK-induced plasma extravasation.

BK-induced plasma extravasation is markedly inhibited twenty-four hours after exposure to the four-day noise stress paradigm (open diamonds, n=8) but is unaffected when measured one hour after the final noise exposure (open triangles, n=14). A curve depicting the plasma extravasation response to BK in home-cage control animals (filled circles, n=18) is reproduced from Figure 15 for comparison.

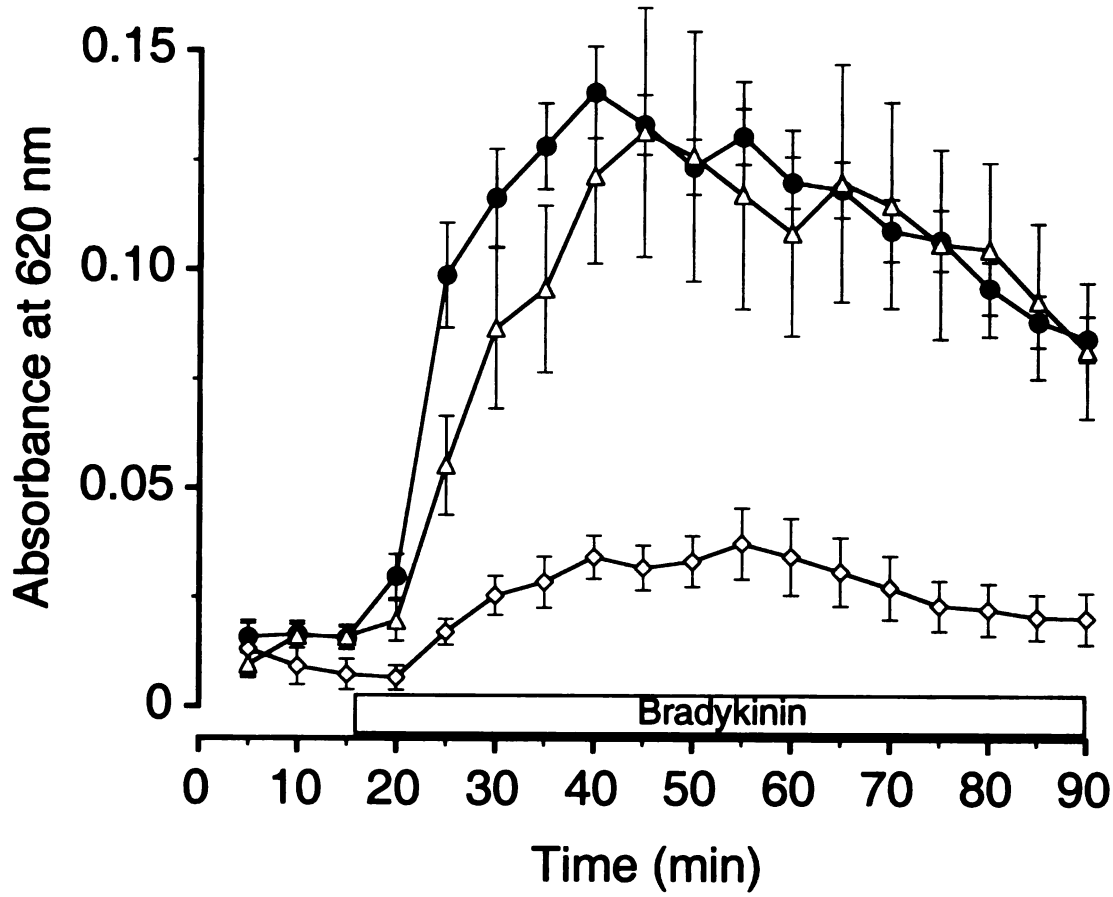

0.15
0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 17. Chronic, intermittent restraint stress potentiates platelet activating factor-induced plasma extravasation.

In contrast to the inhibition of BK-induced plasma extravasation observed in rats exposed to the seven-day restraint stress paradigm (open inverted triangles, n=16), PAF-induced plasma extravasation is potentiated in rats exposed to the seven-day restraint stress paradigm (open triangles, n=16). A curve depicting the plasma extravasation response to PAF in home-cage control animals (filled circles, n=20) is included for comparison.

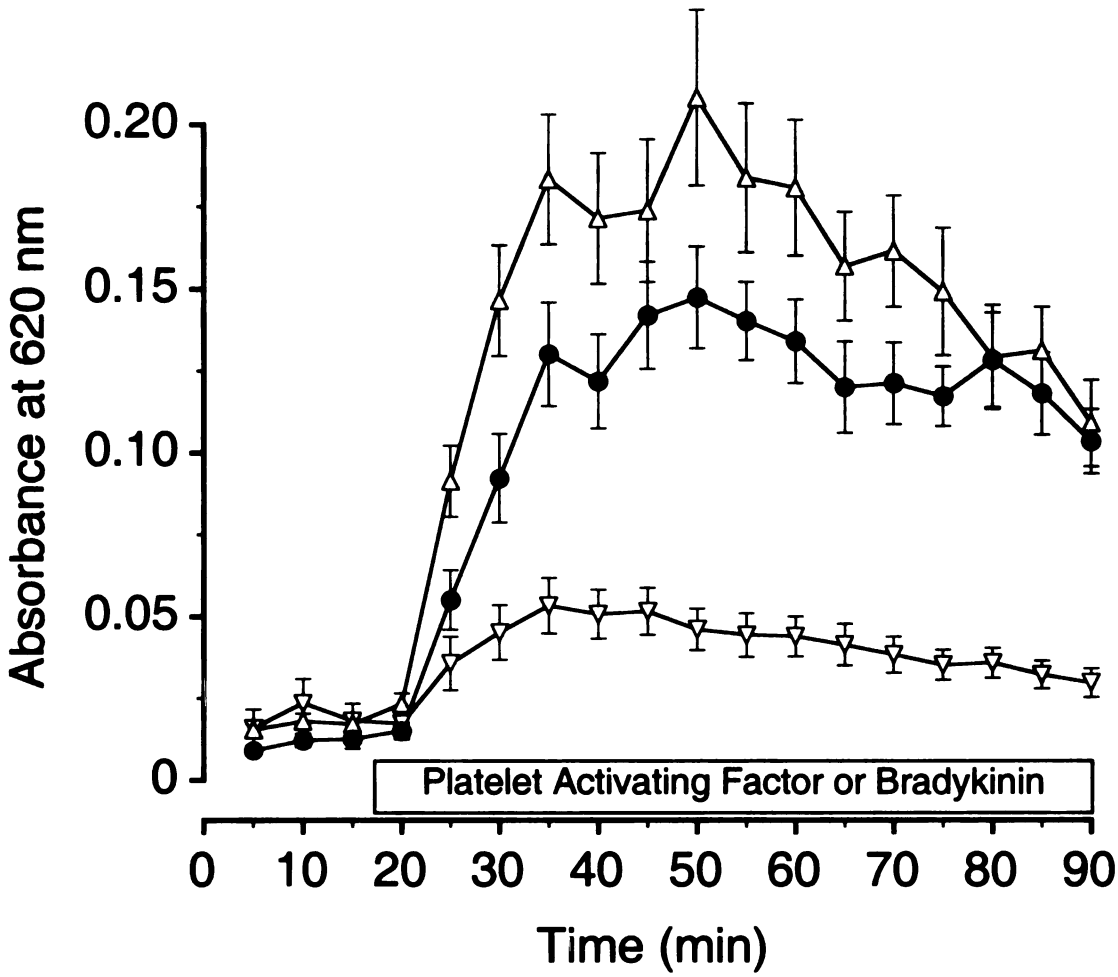
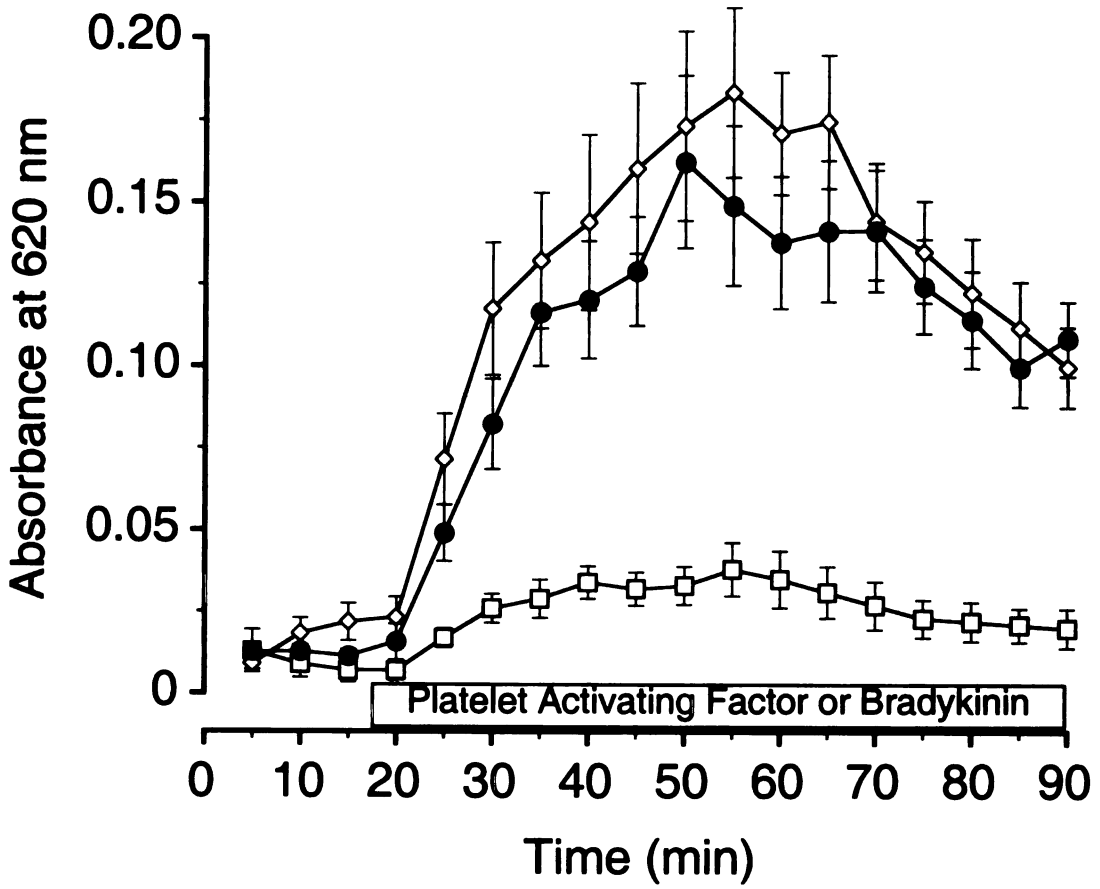

0.20
0.15
0.10
0.05
0
Absorbance at 620 nm
Platelet Activating Factor or Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 18. Chronic, intermittent noise stress potentiates platelet activating factor-induced plasma extravasation.

In contrast to the inhibition of BK-induced plasma extravasation observed in rats exposed to the four-day noise stress paradigm (open squares, n=8), PAF-induced plasma extravasation is potentiated in rats exposed to the four-day sound stress paradigm (open diamonds, n=15). A curve depicting the plasma extravasation response to PAF in home-cage control animals (filled circles, n=20) is reproduced from Figure 17 for comparison.


0.20
0.15
0.10
0.05
0
Absorbance at 620 nm
Platelet Activating Factor or Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

CHAPTER FOUR

CORTICOSTERONE MEDIATES STRESS-INDUCED INHIBITION OF PLASMA EXTRAVASATION

Introduction

In the previous chapter we showed that chronic but not acute stress markedly inhibits BK-induced plasma extravasation. This effect was generalizable as three distinct stressors produced it. The mechanism of stress-induced inhibition of plasma extravasation remains an open question. The finding that stress inhibits neurogenic but not non-neurogenic plasma extravasation suggests that neutrophils and/or sympathetic post-ganglionic nerve terminals may be targets of stress hormones that mediate the effects. Prior to understanding how these targets may be involved in mediating stress-induced inhibition of BK-induced plasma extravasation, it is first necessary to define which stress hormones mediate the inhibition.

Stress induces activation of three circuits: 1.) the HPA axis, 2.) the SA axis and 3.) the sympathetic efferent axis. Stress evokes release of specific hormones form these axes including CRH, ACTH and corticosterone for the HPA axis, epinephrine and opioids for the sympathoadrenal axis and norepinephrine and NPY for the sympathetic efferent axis. Any one or combination of these hormonal mediators may be involved in mediating stress-induced inhibition of plasma extravasation since many of these mediators have been implicated to some extent in the regulation of plasma extravasation (Coderre et al., 1991; Green and Levine, 1992; Karalis et al., 1991; Laue et al., 1988), neutrophil recruitment (Karalis et al., 1991; Yarwood et al., 1993) or blood flow (Berne and Levy, 1993; Corder et al., 1992; White et al., 1990). Regulation of neutrophil recruitment and blood flow are relevant to regulation of plasma extravasation because they both can modulate it (Bjerknes et al., 1991; Bjork et al., 1982; Green et al., 1993; Miao, 1998; Wedmore and Williams, 1981) (refer to

chapter 1 for review of these mediators and their effects on components of the inflammatory response). In this study we determine which neuroendocrine axes and which specific stress hormones mediate restraint stress-induced inhibition of BK-induced plasma extravasation. We chose restraint stress instead of noise stress for further investigation because restraint stress induced inhibition of plasma extravasation without the delay that was observed with noise stress. This delay is likely to add an additional layer of complexity to the mechanism, therefore we deemed the mechanism of inhibition evoked by restraint stress as more appropriate for a first investigation.

Results

Four or seven daily exposures to restraint stress induce inhibition of plasma extravasation.

To determine how many exposures to restraint stress are required to produce inhibition of BK-induced plasma extravasation, we exposed rats to 30 minutes of restraint stress daily for one, four or seven days and then measured BK-induced plasma extravasation one hour after the last stress exposure. BK-induced plasma extravasation was significantly inhibited in rats exposed to four or seven days of restraint stress. (Figure 19; $F(3,48) = 17.3$, $p < 0.001$, post-hoc 4-day or 7-day restraint versus control, $p < 0.01$). As shown previously, BK-induced plasma extravasation in rats exposed to one session of restraint stress was not significantly different from BK-induced plasma extravasation in unstressed, home-cage control rats (Figure 19; $F(3,14) = 19.2$, $p < 0.001$, post-hoc 1-day restraint versus control, ns).

These data indicate that although more than one exposure to stress is required to produce inhibition of plasma extravasation, similar to noise stress, a 4-day exposure period is sufficient to induce the inhibition.

Chronic restraint stress-induced inhibition of plasma extravasation continues for at least five hours after the final exposure to the stressor.

To determine the duration of the restraint stress-induced effects on BK-induced plasma extravasation, we exposed rats to restraint stress daily for seven days and measured BK-induced plasma extravasation one, five or twenty-four hours after cessation of the last stress session. It is important to note that experiments investigating the five-hour post stress time point took place, as did all other experiments, in the trough of the corticosterone circadian rhythm in order to minimize any non-specific effects of circadian rises in corticosterone. BK-induced plasma extravasation was significantly inhibited one and five hours after the cessation of the seventh daily exposure to restraint stress and returned to baseline by twenty-four hours after that exposure (Figure 20; $F(3, 34) = 19.2$, $p < 0.001$, post-hoc 1 hour or 5 hours post restraint versus control or 24 hours post restraint, $p < 0.01$; post-hoc 24 hours post restraint versus home-cage control, ns).

The HPA axis mediates chronic, intermittent restraint stress-induced inhibition of plasma extravasation.

We used surgical interventions to determine which stress circuits (i.e., HPA axis, SA axis or sympathetic efferent axis) mediate the chronic intermittent stress-

induced inhibition of plasma extravasation. To begin to determine the contribution of each stress axis, we lesioned both the HPA and SA axes by removing the adrenal glands and exposing rats to seven days of chronic intermittent restraint stress. BK-induced plasma extravasation was not significantly different between adrenalectomized rats and adrenalectomized rats that had been exposed to chronic, intermittent restraint stress while stressed intact rats showed significantly inhibited plasma extravasation compared to these two groups (Figure 21; $F(3, 48) = 10.6$, $p < 0.001$, post-hoc adrenalectomized versus adrenalectomized plus restraint stress, ns; post-hoc adrenalectomized or adrenalectomized plus restraint stress versus intact plus restraint stress, $p < 0.01$). These data show that chronic, intermittent stress-induced inhibition of plasma extravasation is blocked in adrenalectomized rats and indicates that either or both the HPA and SA axes are involved in mediating the inhibition. It should be noted that BK-induced plasma extravasation was significantly lower in control rats that had been adrenalectomized but that had not been exposed to restraint stress when compared to home-cage intact control rats (Figure 21; $F(3, 48) = 10.6$, $p < 0.001$, post-hoc adrenalectomized versus control rats, $p < 0.01$). It is unclear why adrenalectomy alone induced a small suppression of BK-induced plasma extravasation. These data, however, do not affect the interpretation that adrenalectomy blocks chronic stress-induced suppression of BK-induced plasma extravasation since there is no significant difference in plasma extravasation between adrenalectomized rats and adrenalectomized rats that were exposed to chronic, intermittent restraint.

To distinguish between the contribution of the HPA axis and the SA axis, we surgically denervated the adrenal medullae, leaving the adrenal cortex intact and then

exposed rats to seven days of chronic intermittent restraint stress. Chronic intermittent stress induced a significant inhibition of plasma extravasation in the adrenal medullae-denervated rats that was nearly identical to the stress-induced inhibition observed in intact rats suggesting that the adrenal medullae do not mediate the stress effects (Figure 22; $F(3, 38) = 17.3$, $p < 0.001$, post-hoc adrenal denervated plus restraint stress versus adrenal denervated, $p < 0.01$; post-hoc adrenal denervated plus restraint stress versus intact plus restraint stress, ns). BK-induced plasma extravasation was not significantly different between control rats that had been subjected to adrenal medullae denervation but that had not been exposed to restraint stress and home-cage, intact control rats indicating that adrenal denervation alone had no affect on plasma extravasation (Figure 22; $F(3, 38) = 17.3$, $p < 0.001$, post-hoc adrenal denervated versus home-cage control, ns). Since chronic stress-induced inhibition of plasma extravasation was blocked in adrenalectomized rats but not in adrenal-medullae denervated rats, these data suggest that the HPA axis and specifically the adrenal cortex mediates the inhibitory effect of chronic, intermittent stress on plasma extravasation. Because stress induces rapid synthesis and release of corticosterone from the adrenal cortex, these data suggest corticosterone as a potential mediator of the chronic intermittent stress-induced inhibition of plasma extravasation.

Corticosterone does not directly mediate chronic, intermittent restraint stress-induced inhibition of plasma extravasation.

If corticosterone directly mediates chronic intermittent stress-induced inhibition of plasma extravasation, then plasma corticosterone levels would be

expected to be high when plasma extravasation is inhibited. Therefore, to determine if corticosterone could directly inhibit BK-induced plasma extravasation, we measured plasma corticosterone levels one hour after a single exposure to restraint stress, one hour after the fourth daily exposure to restraint stress and one, five and twenty-four hours after the seventh daily exposure to restraint stress. As shown in Figure 23, plasma corticosterone levels are not associated with BK-induced plasma extravasation. When corticosterone levels are highest (i.e., 1 hour after a single exposure to restraint stress and 1 hour after the fourth daily exposure to restraint stress) in one case (after a single exposure to restraint stress) BK-induced plasma extravasation is similar to control levels but in the other (1 hour after the fourth daily exposure to restraint stress) BK-induced plasma extravasation is inhibited. Similarly, in the two cases in which plasma corticosterone levels are at the lowest levels (i.e., home-cage control and 5 hrs after the seventh daily exposure to restraint stress), in one case (home-cage controls) BK-induced plasma extravasation is normal but in the other (5 hours after the seventh daily exposure to restraint stress) BK-induced plasma extravasation is inhibited. A formal correlation analysis confirms these observations and shows that there is no correlation between plasma corticosterone and BK-induced plasma extravasation (Figure 24; $R = 0.040$, ns).

Repeated daily pulses of corticosterone reproduce chronic, intermittent restraint stress-induced inhibition of plasma extravasation.

Chronic, intermittent but not acute restraint stress inhibits plasma extravasation (Figure 19). An adrenal cortical factor mediates this effect (Figures 21-22) but

corticosterone does not directly induce inhibition of plasma extravasation (Figure 23-24). Therefore, we hypothesized that daily, repeated pulses of corticosterone evoked by stress induce a secondary change in the animal that then, in turn, mediates the inhibition of plasma extravasation. This hypothesis is consistent with our finding that chronic but not acute stress inhibits plasma extravasation. An alternative hypothesis is that a stress-induced adrenal cortical factor other than corticosterone (e.g., interleukin-6 (Zhou et al., 1993)) mediates the stress-induced inhibition of plasma extravasation. To test both of these hypotheses, we blocked the ability of restraint stress to induce production of corticosterone on the first six days of daily restraint stress by administering the corticosterone synthesis inhibitor, metyrapone, prior to the daily stress session. Metyrapone was not administered prior to the seventh daily restraint session so that, in terms of corticosterone secretion, these animals would be expected to be similar to acutely stressed animals but in terms of HPA axis functions not affected by corticosterone they would be expected to be similar to chronically stressed animals. In this way we were able to determine the effect of repeated corticosterone pulses on plasma extravasation and to test for a ossible contribution of adrenal cortical factors other than corticosterone.

As shown in Figure 25 (inset), metyrapone treatment affected corticosterone production as expected since chronically stressed animals that had been treated with metyrapone for 6 days exhibited levels of corticosterone on day seven that were not significantly different from those observed in acutely stressed animals ($F(4,33) = 6.4$, $p < 0.001$, post-hoc, ns) but that were significantly enhanced compared to animals subjected to chronic stress without metyrapone treatment ($F(4,33) = 6.4$, $p < 0.001$,

post-hoc, $p < 0.05$). Metyrapone treatment alone evoked corticosterone levels that were not significantly different from levels measured in home-cage control animals ($F(4,33) = 6.4$, $p < 0.001$, post-hoc, ns).

Animals treated with metyrapone prior to their daily exposure to stress did not show stress-induced inhibition of BK-induced plasma extravasation but instead exhibited similar levels of BK-induced plasma extravasation to animals treated with metyrapone and not subjected to restraint stress (Figure 25; $F(4,61) = 11.3$, $p < 0.001$; post hoc, ns). Animals exposed to chronic, intermittent restraint stress showed inhibition of BK-induced plasma extravasation when compared to both home-cage controls and to animals treated with metyrapone plus chronic, intermittent restraint stress (Figure 25; $F(4,61) = 11.3$, $p < 0.001$; post hoc, $p < 0.01$). These data suggest that repeated daily pulses of corticosterone are required to produce inhibition of plasma extravasation. This experiment also excludes other possible adrenal cortical factors, induced by stimulation of the HPA axis (e.g., interleukin-6 (Zhou et al., 1993)), as mediators of these effects.

As shown in Figure 25, metyrapone treatment alone produces a significant inhibition of BK-induced plasma extravasation (Figure 5A, $F(4,61) = 11.3$, $p < 0.001$; post hoc home-cage control versus metyrapone alone, $p < 0.01$), which may be due to its ability to dramatically decrease blood pressure which non-specifically inhibits plasma extravasation (Miao, 1998). For this reason we used an additional experimental approach to confirm the finding that repeated daily pulses of corticosterone induce inhibition of plasma extravasation. ACTHar® (4 units/kg), a slow-release form of ACTH, induces a sustained rise in plasma corticosterone in the

rat that is not significantly different in level or in time course to that induced by restraint stress (Dallman and Jones, 1973). Therefore, we tested the hypothesis that daily pulses of corticosterone, similar to those evoked by restraint stress, are sufficient to produce inhibition of BK-induced plasma extravasation by injecting ACTHar® (4 units/kg) daily for seven days. Consistent with previous work (Dallman and Jones, 1973), in anesthetized animals, ACTHar® (4 units/kg) produced similar levels of plasma corticosterone as seven-day restraint stress and both of these groups showed significantly higher corticosterone levels than vehicle-injected or home-cage control animals (Figure 26, inset, $F(3,22) = 19.3$, $p < 0.001$; post-hoc ACTHar® versus 7-day restraint, ns; post-hoc ACTHar® or 7 day restraint versus vehicle or home-cage control, $p < 0.01$). As shown in Figure 26, ACTHar® injections (4 units/kg) were sufficient to produce a similar inhibition of BK-induced plasma extravasation as that produced by seven-day restraint stress. BK-induced plasma extravasation was unaffected in vehicle controls and was similar to BK-induced plasma extravasation observed in home-cage controls ($F(3,37) = 12.9$, $p < 0.001$; post-hoc ACTHar® versus 7-day restraint, ns; post-hoc vehicle versus control, ns; post-hoc ACTHar® or 7 day restraint versus vehicle or home-cage control, $p < 0.01$). These data indicate that daily pulses of corticosterone evoked by stressful stimulation mediate the observed inhibition of synovial plasma extravasation.

Discussion

We have shown that four or seven repeated exposures to restraint stress markedly inhibit BK-induced plasma extravasation, an integral component of the

inflammatory response, while a single exposure to the same stressor does not affect BK-induced plasma extravasation. This inhibition is not transient as it lasts at least five hours after exposure to the stressor. Our data further show that the observed stress-induced inhibition of plasma extravasation is mediated by the HPA axis and specifically by corticosterone. Corticosterone does not, however, mediate stress-induced inhibition of plasma extravasation in a simple, direct way since plasma extravasation is not correlated with plasma corticosterone levels and since repeated exposure to stress levels of corticosterone are required to induce the inhibition.

The mechanism by which corticosterone inhibits plasma extravasation in this model remains to be established. Corticosterone may inhibit plasma extravasation by induction of a secondary factor(s). The data from this study indicate that a delay of more than two hours would be required to induce this factor since no effect on plasma extravasation is apparent during the first two hours after one exposure to the stressor. Glucocorticoids are known to induce upregulation of β_2-adrenergic receptors (Bronnegard et al., 1995; Mak et al., 1995; Steinkraus et al., 1996) which play a role in the regulation of BK-induced plasma extravasation (Coderre et al., 1991). Glucocorticoid-induced upregulation of β_2-adrenergic receptors requires *de novo* protein synthesis (Fraser and Venter, 1980) and requires more than two hours for induction (Mak et al., 1995). Of note, when glucocorticoids are administered *in vivo*, β_2-adrenergic receptor upregulation does not occur in response to one injection of glucocorticoids but rather repeated daily injections of glucocorticoids are required to induce β_2-adrenergic receptor upregulation (Mak et al., 1995). An alternative hypothesis is that repeated exposure to stress-induced elevations in plasma

corticosterone, may alter the HPA axis such that subsequent stress produces effects that are not observed in naïve animals. For example, chronically but not acutely stressed rats show decreased expression of arginine vasopressin (AVP) in the amygdaloid nuclei (Albeck et al., 1997). This decreased AVP expression is strongly correlated with decreased plasma testosterone levels (Albeck et al., 1997) and is consistent with the observation that chronic but not acute stress decreases levels of plasma testosterone (Batuman et al., 1990). This may be relevant to the mechanism of stress-induced inhibition of plasma extravasation since plasma testosterone is correlated with level of BK-induced plasma extravasation (Green et al., in press).

In summary, this study has defined the neuroendocrine circuit and the specific stress hormone that mediates restraint stress-induced inhibition of plasma extravasation. Further investigation into the cellular mechanism of stress-induced inhibition of plasma extravasation is required to determine the targets of corticosterone action. An investigation of the cellular mechanisms of stress-induced inhibition of plasma extravasation is the subject of the next chapter.

Figure 19. Four or seven daily exposures to restraint stress induce inhibition of plasma extravasation.

BK induces sustained plasma extravasation (filled circles, n = 14). One exposure to restraint stress does not affect BK-induced plasma extravasation (open squares, n=13) while four (open diamonds, n=12) or seven (open, inverted triangles, n=14) daily exposures to restraint stress markedly inhibit it.

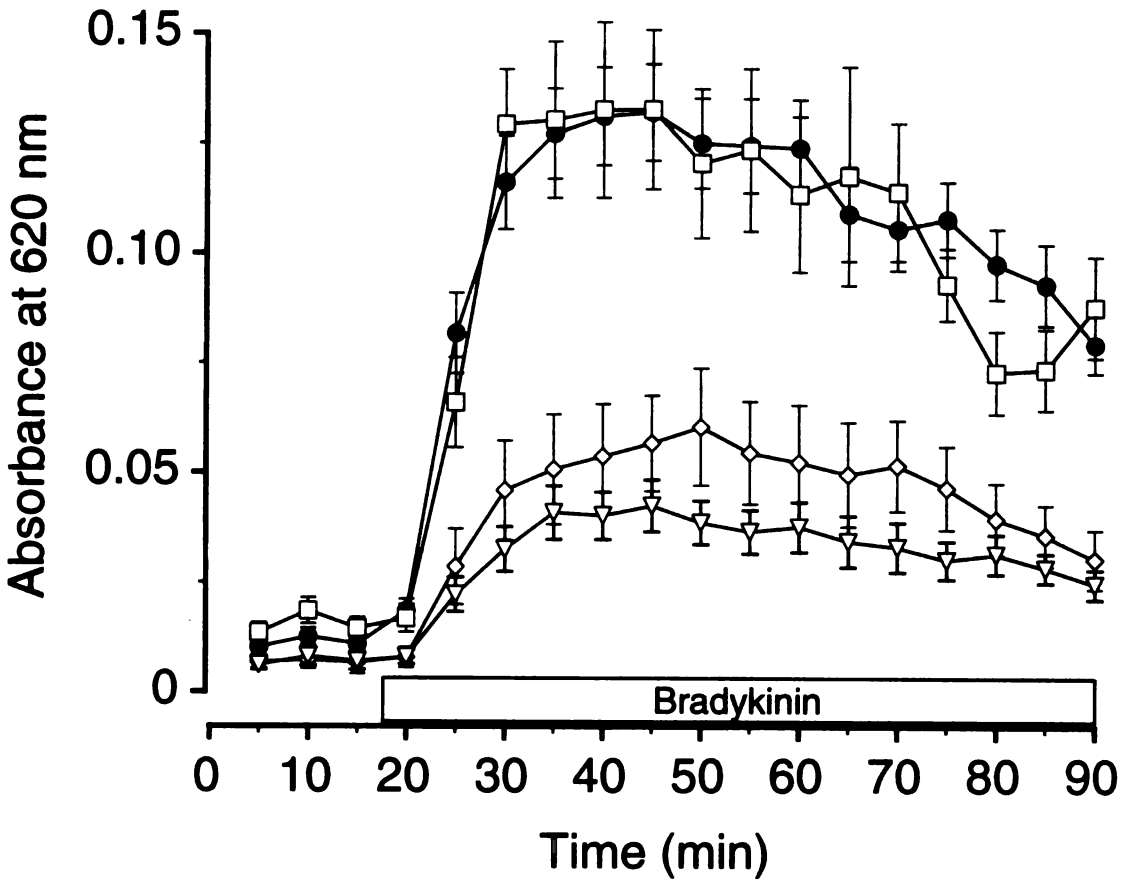

0.15
0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 20. Chronic, intermittent restraint stress-induced inhibition of plasma extravasation continues for at least five hours after the final exposure to the stressor.

BK-induced plasma extravasation is markedly inhibited one (inverted triangles, n = 14) and five hours (squares, n = 5) after the seventh daily exposure to restraint stress and returns to baseline 24 hours later (triangles, n = 5). A curve depicting the plasma extravasation response to BK in home-cage control animals (filled circles, n=14) is reproduced from Figure 19 for comparison.

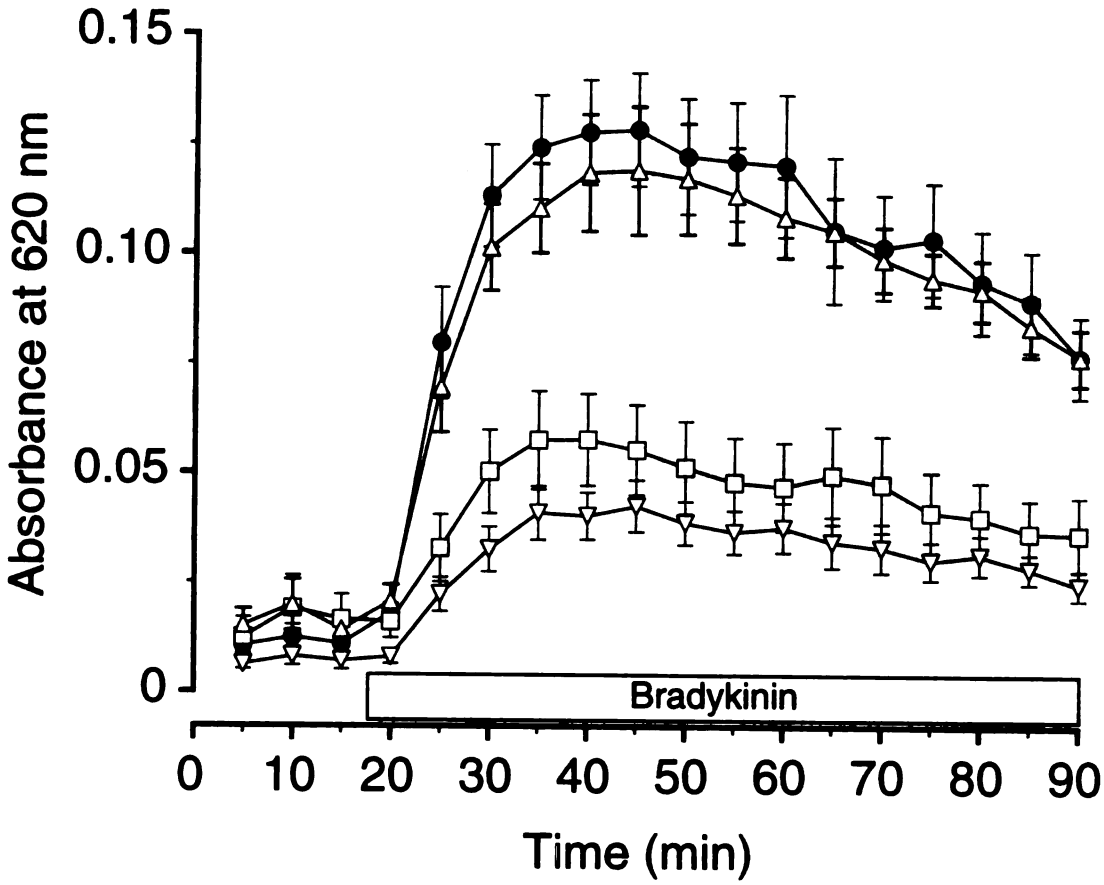

0.15
0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 21. Restraint stress-induced inhibition of plasma extravasation is blocked by adrenalectomy.

Chronic restraint stress-induced inhibition of plasma extravasation (filled triangles, n=14) was completely blocked in animals that had been adrenalectomized one week prior to receiving restraint stress (open, inverted triangles, n =11) since BK-induced plasma extravasation was not significantly different in these animals compared to that in animals that had been adrenalectomized but that were not subjected to restraint stress (open circles, n = 15). Curves depicting the plasma extravasation response to BK in home-cage control animals (filled circles, n=14) and in intact animals subjected to 7-day restraint stress (filled, inverted triangles, n=14) are reproduced from Figure 19 for comparison.

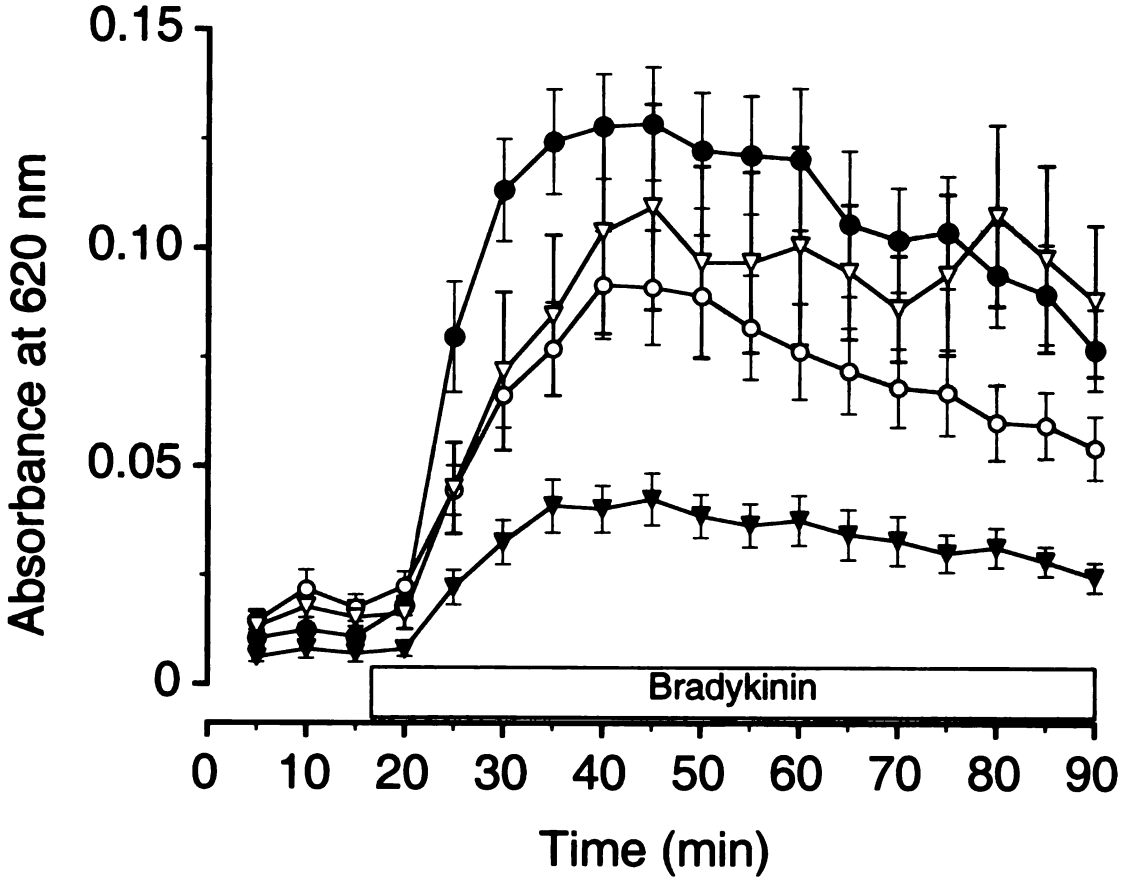

0.15
0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 22. Adrenal medullae denervation does not affect restraint stress-induced inhibition of plasma extravasation.

Chronic restraint stress-induced inhibition of plasma extravasation (filled, inverted triangles, n=14) was not affected in animals that had undergone adrenal medullae denervation one week prior to receiving restraint stress (open, inverted triangles, n =10) since BK-induced plasma extravasation was not significantly different in these groups. Adrenal denervation alone (open circles, n=6) had no effect on BK-induced plasma extravasation as animals with denervated adrenal medullae exhibited similar BK-induced plasma extravasation as intact home-cage controls (filled circles, n=14). Curves depicting the plasma extravasation response to BK in home-cage control animals and in intact animals subjected to 7-day restraint stress are reproduced from Figure 19 for comparison.

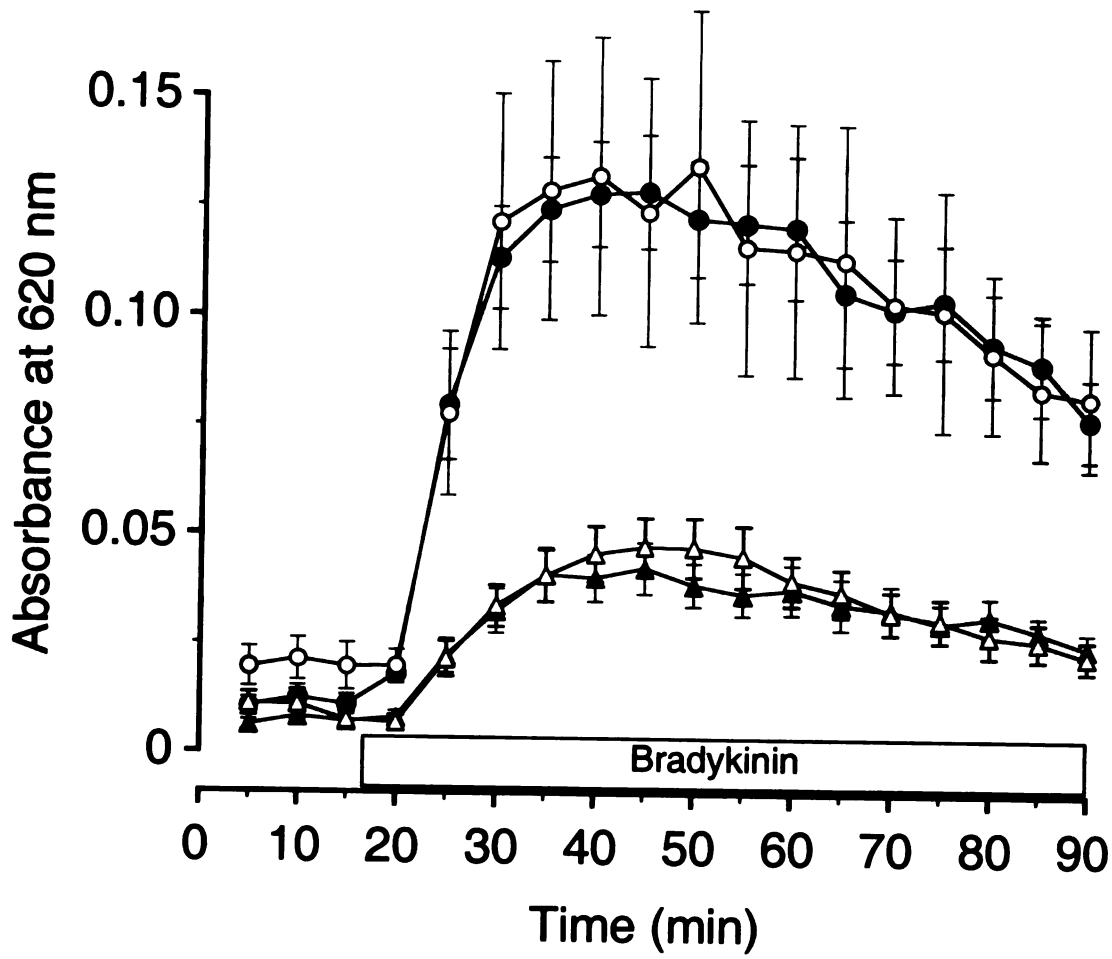

0.15
0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 23. Plasma corticosterone plotted against plasma extravasation.

Plasma corticosterone (gray bars) was measured in separate groups of anesthetized animals one hour after exposure to one (1 day, n=8) four (4 day, n=5) or seven (7 day, n=7) daily restraint sessions and 5 (7 day + 5 hrs, n=5) and 24 hours (7 day + 24 hrs, n=10) after the seventh daily exposure to restraint. Plasma corticosterone in anesthetized home-cage control animals (Control, n=10) is also included. BK-induced plasma extravasation (black bars) was measured in these same groups of rats. An average of BK-induced plasma extravasation was computed for each rat by averaging the plasma extravasation values after administration of BK (time points 20 min through 90 min). Visual examination of the data reveals no apparent relationship between plasma corticosterone levels and levels of plasma extravasation.

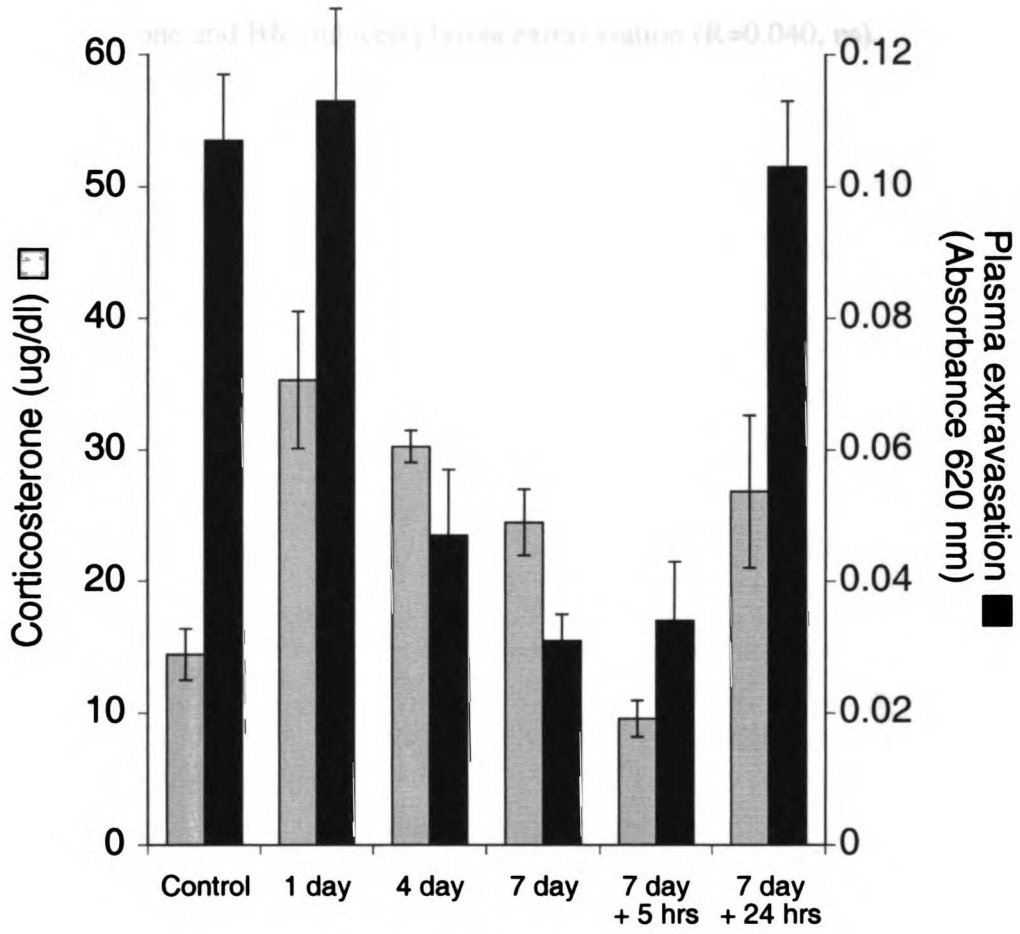

Corticosterone (ug/dl) □
Plasma extravasation ■ (Absorbance 620 nm)
60
50
40
30
20
10
0
0.12
0.10
0.08
0.06
0.04
0.02
0
Control
1 day
4 day
7 day
7 day + 5 hrs
7 day + 24 hrs

Figure 24. Plasma corticosterone does not correlate with bradykinin-induced knee joint plasma extravasation.

A Pearson product-moment correlation coefficient was computed to determine the relationship between plasma corticosterone and mean plasma extravasation. This analysis indicated that there is no significant correlation between plasma corticosterone and BK-induced plasma extravasation (R=0.040, ns).

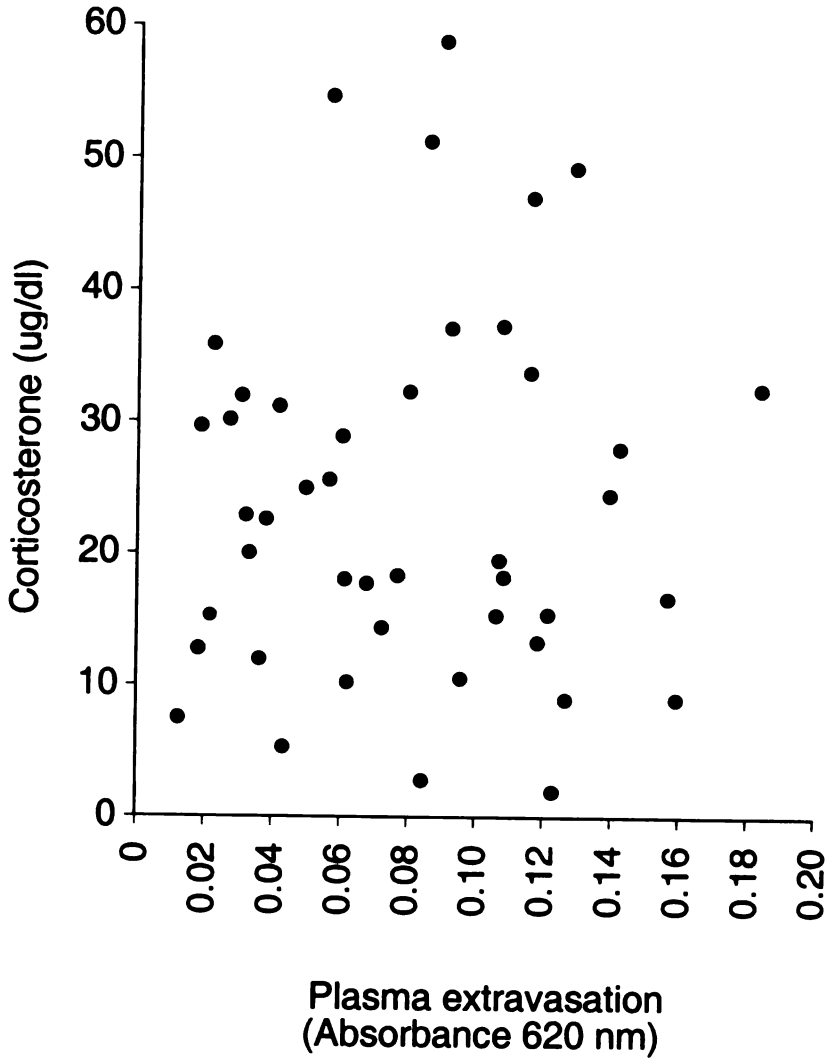

Corticosterone (ug/dl)
60
50
40
30
20
10
0
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
Plasma extravasation
(Absorbance 620 nm)

Figure 25. Restraint stress-induced inhibition of plasma extravasation is blocked by metyrapone treatment.

Inset: Animals treated with the corticosterone synthesis inhibitor, metyrapone, prior to restraint stress on the first six days of the seven daily exposures (R-m, n=6) showed plasma corticosterone levels that were not significantly different than those exhibited in untreated animals exposed to only one restraint session (R-a, n=8). Metyrapone treatment alone (M, n=7) evoked plasma corticosterone levels similar to those observed in animals exposed to seven days of restraint stress (R-c, n= 7). Plasma corticosterone in home-cage control animals (C, n=10) is reproduced from Figure 23 for comparison.

Chronic restraint stress-induced inhibition of plasma extravasation (filled, inverted triangles, n=14) was completely blocked in animals that had been treated with metyrapone, on the first six days of the seven daily exposures to restraint stress (open, inverted triangles, n=11) since BK-induced plasma extravasation was not significantly different in these animals compared to that in animals not exposed to restraint stress that had been treated daily for six days with metyrapone (open circles, n=13). Curves depicting the plasma extravasation response to BK in home-cage control animals (filled circles, n=14) and in intact animals subjected to 1-day (filled squares, n=13) or 7-day restraint stress (filled, inverted triangles, n=14) are reproduced from Figure 19 for comparison.

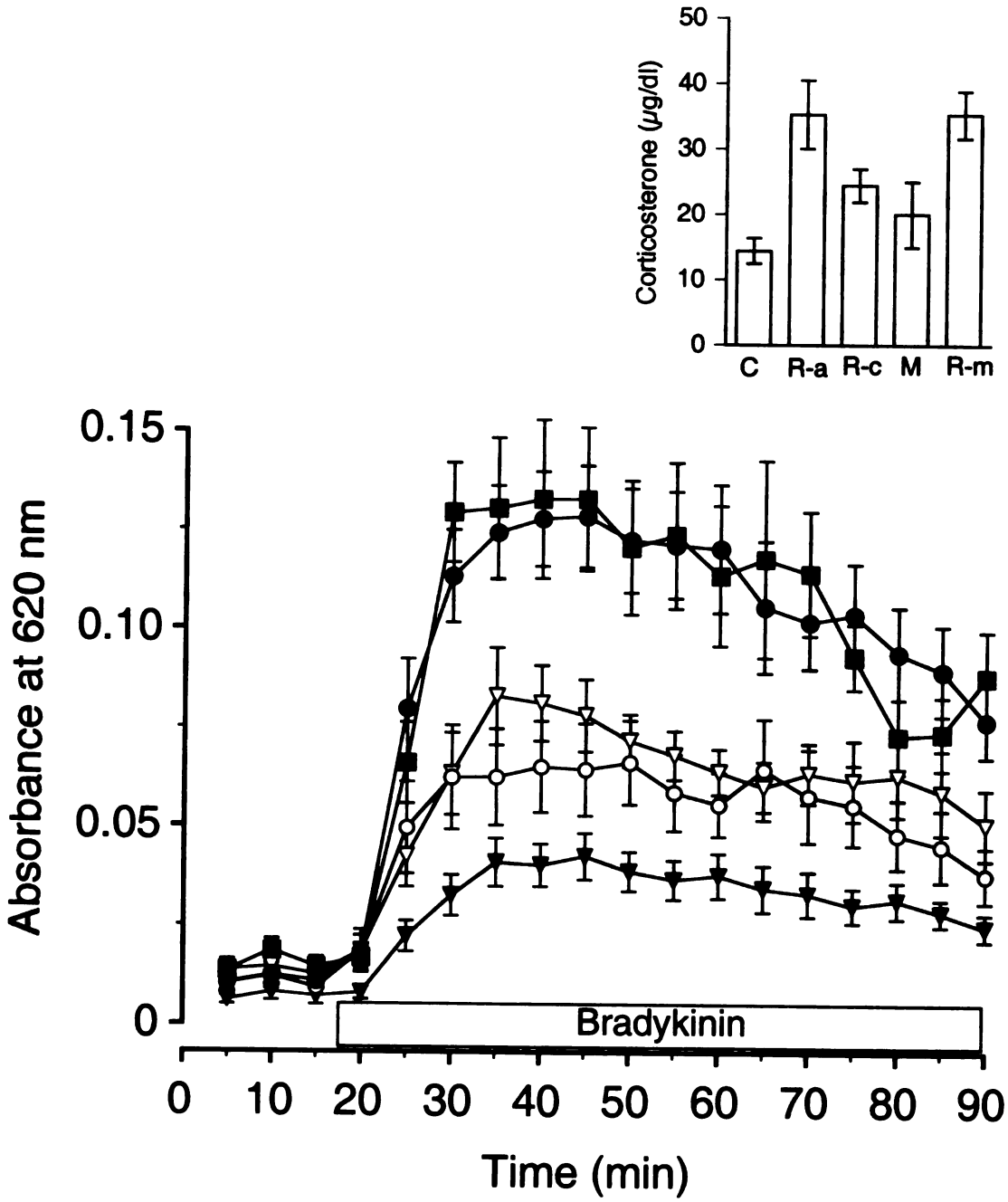
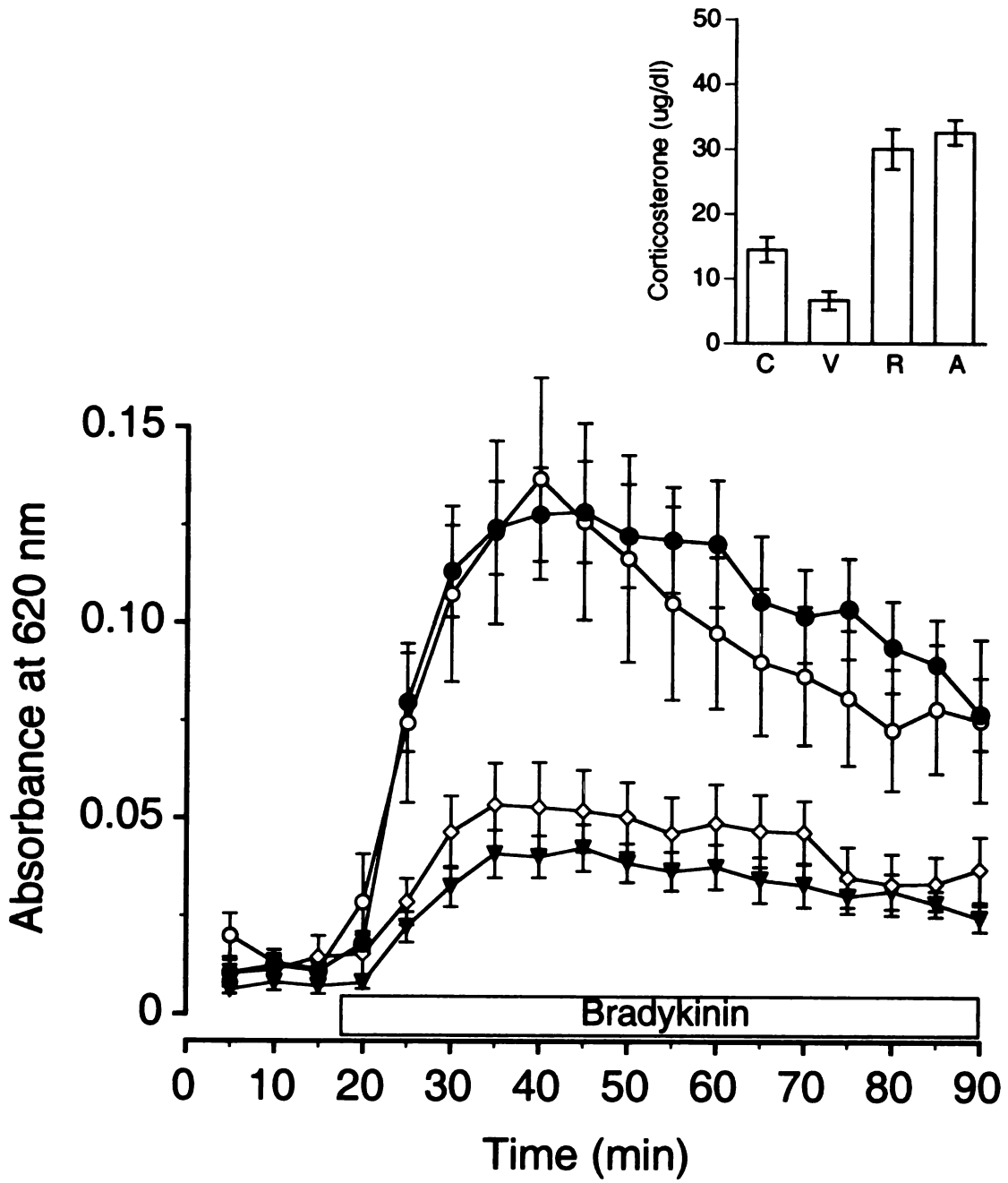

Corticosterone (μg/dl)
50
40
30
20
10
0
C
R-a
R-c
M
R-m
Absorbance at 620 nm
0.15
0.10
0.05
0
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 26. Repeated daily ACTHar® injections reproduce chronic, intermittent restraint stress-induced inhibition of plasma extravasation.

Inset: Animals injected with ACTHar® daily for seven days (A, n=6) showed plasma corticosterone levels that were not significantly different than those exhibited by animals exposed to seven daily sessions of restraint stress (R, n=7). Plasma corticosterone was significantly elevated in these groups compared to control animals injected daily with saline vehicle (V, n=3). Plasma corticosterone levels in home-cage control animals (C, n=10) are reproduced from Figure 23 for comparison.

Chronic restraint stress-induced inhibition of plasma extravasation (filled, inverted triangles, n=14, reproduced from figure 19) was mimicked by daily injections of ACTHar® (open diamonds, n=8) since BK-induced plasma extravasation was not significantly different in these two groups. Furthermore, BK-induced plasma extravasation was significantly inhibited in both of these groups when compared to BK-induced plasma extravasation in animals injected daily with saline vehicle (open circles, n=5). The injection procedure alone did not affect BK-induced plasma extravasation because BK-induced plasma extravasation was not significantly different in animals that had been injected daily with saline vehicle (open circles, n=5) and in home-cage controls (filled circles, n=14, reproduced for comparison from Figure 19).


Corticosterone (ug/dl)
50
40
30
20
10
0
C
V
R
A
Absorbance at 620 nm
0.15
0.10
0.05
0
Bradykinin
0
10
20
30
40
50
60
70
80
90
Time (min)

CHAPTER FIVE

ACTIVATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS INHIBITS PLASMA EXTRAVASATION BY INHIBITING NEUTROPHIL RECRUITMENT

Introduction

In the previous chapter we investigated the neuroendocrine mechanism of restraint stress-induced inhibition of plasma extravasation. We found that inhibition of plasma extravasation is mediated by the HPA axis and specifically by corticosterone. The local cellular mechanism of restraint stress-induced inhibition of plasma extravasation, however, remains an open question. The finding that restraint stress inhibits neurogenic but not non-neurogenic plasma extravasation suggests that neutrophils and/or sympathetic post-ganglionic efferents may play a role in this mechanism since neutrophils and sympathetic post-ganglionic efferents are required for induction and maintenance of neurogenic inflammation but not for non-neurogenic inflammation (Green et al., 1993).

Acute inflammation is characterized by increased blood flow, plasma extravasation, leukocyte infiltration and pain. Neutrophils, the first leukocytes to be recruited to sites of inflammation, accumulate in response to appropriate inflammatory signals from tissue. Circulating neutrophils initially tether to and slowly roll on the endothelium. The cells then firmly adhere, arrest and finally transmigrate toward the inflammatory stimulus. On neutrophils, initial tethering and rolling events are mediated in part, by the leukocyte adhesion molecule, L-selectin (Smith et al., 1991). L-selectin-mediated neutrophil adhesion is prevented when L-selectin is shed from the neutrophil membrane (Jutila et al., 1991; Kishimoto et al., 1989) through cleavage by a cell surface enzyme (Preece et al., 1996) recently identified as TACE (Peschon et al., 1998). Although L-selectin shedding is recognized as a potentially important mechanism for regulating neutrophil function and several laboratories have attempted

to elucidate a function for L-selectin shedding, (Allport et al., 1997; Alon et al., 1998; Jutila et al., 1989; Kishimoto et al., 1989; Walcheck et al., 1996), an *in vivo* physiological function for L-selectin shedding has not yet been demonstrated.

Blocking L-selectin function by systemic infusion of anti-L-selectin monoclonal antibodies (Mulligan et al., 1995) or by *in vitro* induction of L-selectin shedding (Jutila et al., 1991) inhibits neutrophil accumulation *in vivo*. Therefore, *in vivo* shedding of L-selectin would be expected to inhibit neutrophil accumulation at inflammatory sites. This shedding might also inhibit plasma extravasation since neutrophils are necessary for induction of plasma extravasation in many inflammatory models (Bjerknes et al., 1991; Bjork et al., 1982; Green et al., 1993; Wedmore and Williams, 1981). We hypothesized that stress inhibits plasma extravasation by inducing *in vivo* shedding of L-selectin from neutrophils.

To test this hypothesis we used an acute model of HPA-axis mediated inhibition of plasma extravasation(Green et al., 1995). In this model, noxious electrical stimulation of sensory nerve fibers in the hindpaw induces a rapid and robust inhibition of BK-induced plasma extravasation (Figure 27). We used this acute model for these studies because it allows the use of within subjects controls. Due to the high level of biological variability between animals and the potential of small effect sizes, we wished to use a within subjects control to reduce our signal to noise ratio and thereby maximize our chance of observing small effects. We chose the noxious stimulation model because it is similar in key ways to the chronic restraint stress model. Like chronic restraint stress-induced inhibition of plasma extravasation, noxious stimulation-induced inhibition of plasma extravasation is mediated by the

HPA axis (Green et al., 1995) and specifically by corticosterone (Green et al., 1998). An additional important similarity is that in both models only neurogenic (BK-induced) and not non-neurogenic (PAF-induced) plasma extravasation is inhibited (Green et al., 1995).

Results

Noxious stimulation inhibits plasma extravasation.

When perfused continuously through the knee joint BK induces rapid onset and sustained plasma extravasation (Figure 28). Consistent with our previous results (Green et al., 1995), noxious, but not non-noxious, stimulation of the hindpaw induced a robust inhibition of BK-induced plasma extravasation in the knee joint (Figure 28; $F(2,27) = 5.26$. $p < 0.05$; post-hoc BK + noxious stimulation versus BK + non-noxious stimulation or BK alone, $p < 0.01$; post-hoc BK + non-noxious stimulation versus BK alone, $p < 0.01$).

Bradykinin-induced plasma extravasation is neutrophil dependent.

While previous data from our laboratory suggest that neutrophils contribute to the induction of BK-induced plasma extravasation, the methods used to determine this were relatively crude (Bjerknes et al., 1991; Green et al., 1993). In these studies the treatments did not selectively inhibit neutrophil accumulation but rather inhibited accumulation of all leukocytes at the knee joint. Therefore, to determine if bradykinin-induced plasma extravasation in the knee joint is neutrophil dependent, we measured plasma extravasation in animals that had been treated with neutrophil-

depleting antiserum (Johnston et al., 1996). This serum depleted circulating neutrophils by approximately 90% (measured by differential counts of blood smears at 20 and 40 minutes after administration; Figure 29). Monocyte counts were not affected by the serum (data not shown). Lymphocyte counts increased in the serum-treated animals at 20 and 40 minutes after administration of the neutrophil depleting serum ($F(1,8) = 9.2$, $p < 0.05$). However, since cell counts were performed as differential counts, and lymphocytes compose the vast majority of rat leukocytes, this increase is likely to only reflect the decrease in neutrophils and not an increase in lymphocytes. Neutrophil numbers began to recover approximately 60 minutes after injection of the serum.

BK-induced plasma extravasation was significantly inhibited in neutrophil-depleted animals indicating that BK-induced plasma extravasation in the knee joint is neutrophil dependent (Figure 30; $F(1,12) = 5.05$, $p < 0.05$). Interestingly, the magnitude of the decrease in BK-induced plasma extravasation exhibited by the neutrophil depleted animals was similar to that produced by noxious stimulation (Figure 28). This observation supports the hypothesis that inhibition of neutrophil recruitment mediates noxious stimulation-induced inhibition of plasma extravasation.

Noxious stimulation inhibits neutrophil accumulation at the knee joint

To determine if noxious stimulation affects neutrophil accumulation at the knee joint, in a blinded study, numbers of infiltrating neutrophils were assessed in rat knee joints that had been continuously perfused with saline or BK (Figure 31). These numbers were compared with counts of neutrophils in knee joints from rats that had

received noxious stimulation of the hindpaw in addition to BK perfusion of the knee joint (Figure 31). Bradykinin induced more than a 3-fold increase in neutrophil accumulation compared to saline. This increase was completely abolished in rats that had also received noxious stimulation (Figure 31; $F(2,23) = 3.67$, $P < 0.05$; post-hoc comparisons BK versus saline or BK + noxious stimulation, $p < 0.05$; post-hoc comparison BK + noxious stimulation versus saline, ns).

Systemic block of L-selectin inhibits plasma extravasation.

Noxious stimulation inhibits both BK-induced plasma extravasation and neutrophil accumulation in the knee joint (Figures 28, 31, 32). Since L-selectin mediates initial adherence events by neutrophils (Smith et al., 1991) and since BK-induced plasma extravasation is neutrophil-dependent, we hypothesized that inhibition of L-selectin expression might mediate the noxious stimulation-induced decrease in neutrophil accumulation and thereby inhibit plasma extravasation in this acute model of inflammation. To determine if blocking of L-selectin affects BK-induced plasma extravasation, we established BK-induced plasma extravasation and then administered systemically an L-selectin blocking antibody (HRL-3) that has been shown to substantially inhibit leukocyte tethering (Johnston et al., 1996) and neutrophil migration *in vivo* (Mulligan et al., 1995). Rats treated with an anti-L-selectin monoclonal antibody (HRL-3, $F(ab)'_2$ fragments), but not those treated with an isotype-matched control antibody (hamster IgG, $F(ab)'_2$ fragments), exhibited a pronounced inhibition of BK-induced plasma extravasation (Figure 33; $F(1,19) = 11.06$, $P < 0.01$). This inhibition of BK-induced plasma extravasation was similar in

time course and in magnitude to that induced by noxious stimulation (Figure 28), suggesting that noxious nerve stimulation may inhibit plasma extravasation though an L-selectin-dependent mechanism.

Noxious nerve stimulation induces L-selectin shedding from circulating neutrophils.

We next tested the hypothesis that the inhibitory effect of noxious stimulation is mediated via shedding of L-selectin from circulating neutrophils by measuring L-selectin levels on neutrophils from animals that had been exposed to noxious and non-noxious electrical stimulation of the hindpaw (Figure 34). An average of several experiments indicates that L-selectin expression on neutrophils is significantly decreased after noxious but not after non-noxious stimulation (Figure 35; $t(9) = 4.2$, $P < 0.01$). Specifically, the numbers of neutrophils expressing detectable levels of L-selectin decreased by 62% when compared to levels measured in the same animals during BK-induced plasma extravasation prior to noxious stimulation (Figure 35). The neutrophils that remained positive for L-selectin did not show a change in mean fluorescence indicating that a sub-population of neutrophils is resistant to the induced L-selectin shedding in this model. This pattern of L-selectin shedding as a complete loss from a population of neutrophils is consistent with that reported by others when neutrophils were exposed *in vitro* to leukotriene B_4 (Jutila et al., 1989).

L-selectin shedding from neutrophils, induced by noxious nerve stimulation, inhibits in vitro tethering and rolling of these cells.

To assess the functional impact of the observed noxious stimulus-induced shedding of L-selectin, we isolated neutrophils from rats that had received either noxious or non-noxious stimulation and compared the tethering and rolling behavior of these cells in an *in vitro* rolling chamber. We used endothelial ligands, peripheral node addressin (PNAd) isolated from human tonsils, as the substrate for rolling because PNAd supports L-selectin-mediated rolling of neutrophils (Lawrence et al., 1995). Neutrophils isolated from rats exposed to the noxious stimulus showed a 59% reduction in tethering and rolling compared to neutrophils isolated from control rats (non-noxious stimulus; Figure 36; $t(15) = 4.2$, $p < 0.001$). Rolling was blocked by a carbohydrate antagonist (i.e., fucoidan) and by EDTA, confirming that it was mediated by L-selectin (Rosen and Bertozzi, 1994). For cells that did tether, there was no difference in rolling velocity or in binding strength with the substrate, indicating that L-selectin retained by these cells was functional (data not shown).

Blocking L-selectin shedding blocks noxious stimulation-induced inhibition of plasma extravasation.

We used a TACE inhibitor, Ro31-9790, to determine whether blocking of L-selectin shedding would prevent noxious stimulation-induced inhibition of plasma extravasation. Ro31-9790 is a hydroxamic acid-based inhibitor of zinc-dependent matrix metalloproteinases, which inhibits L-selectin shedding (Allport et al., 1997; Preece et al., 1996). Prior to noxious stimulation, the BK-induced plasma extravasation response was similar to that observed in untreated animals (Figure 37; $F(1,15) = 2.26$, ns). However, in the Ro31-9790 treated animals, noxious stimulation

did not produce the characteristic decrease in BK-induced plasma extravasation indicating that shedding of L-selectin mediates this phenomenon (Figure 37; ($F(2,26) = 6.32$, $p < 0.01$; post-hoc comparison noxious stimulation versus noxious stimulation + Ro31-9790, ns). To confirm the efficacy of the sheddase inhibitor, we analyzed L-selectin expression on neutrophils from a group of rats treated with Ro31-9790. In contrast to the marked reduction of L-selectin expression on neutrophils from rats exposed to the noxious stimulus, (Figure 35), there was no observable loss of L-selectin expression on neutrophils isolated from rats treated with the sheddase inhibitor prior to noxious stimulation (Table 1). That is, L-selectin expression on neutrophils from rats treated with the sheddase inhibitor was similar to expression on neutrophils isolated from control rats exposed to the non-noxious stimulus.

Discussion

The data presented in this study suggest that inhibition of plasma extravasation, produced by noxious stimulation-induced activation of the HPA axis, is mediated by inhibition of neutrophil recruitment. Specifically, we have shown that BK-induced plasma extravasation is neutrophil dependent and that noxious stimulation inhibits neutrophil recruitment. We have further shown that blocking of L-selectin inhibits plasma extravasation, that noxious stimulation induces L-selectin shedding from circulating neutrophils and that this shedding results in inhibition of tethering and rolling by these cells. Finally, we have shown that blocking noxious stimulation–induced L-selectin shedding blocks noxious stimulation-induced inhibition of plasma extravasation.

It remains an open question whether inducible L-selectin shedding is also the mechanism by which chronic stress inhibits plasma extravasation. The large decrement in L-selectin expression observed in this study may make it possible to investigate this in the chronic stress model. This investigation will be complicated, however, by the fact that new neutrophils are continually released from the bone marrow.

An interesting side note to this investigation is that activation of sensory neurons by the procedure used in this model is similar to activation that is induced by inflammatory pain. In fact, this model has been used to demonstrate systemic feedback control of the inflammatory response since activation of pain fibers in the hindpaw inhibits plasma extravasation at a distant site, the knee joint (Green et al., 1995). Therefore, this noxious stimulation-induced shedding of L-selectin may provide a mechanism by which the organism controls ongoing inflammatory responses. For example, L-selectin has recently been implicated in leukocyte-leukocyte interactions (Alon et al., 1996), therefore L-selectin shedding could modulate inflammation throughout its course by decreasing secondary tethers between neutrophils that are already adhered to the venular endothelium and other leukocytes. We suggest then that negative control of the inflammatory response is the long sought function of *in vivo* L-selectin shedding.

Identification of the hormonal mediator that directly induces the L-selectin shedding observed in this model remains a topic for further study. This hormone, however, is likely to be derived from the HPA axis since noxious stimulation-induced inhibition of plasma extravasation is dependent on the HPA axis. There is some

evidence that glucocorticoids can induce L-selectin shedding. For example, *in vivo* treatment with glucocorticoids decreases L-selectin expression on neutrophils (Burton et al., 1995; Jilma et al., 1997). However, *in vitro* studies have failed to demonstrate an effect of glucocorticoids on L-selectin expression by resting neutrophils (Diaz-Gonzalez et al., 1995; Filep et al., 1997). This discrepancy may indicate that glucocorticoids indirectly modulate L-selectin expression. For example, glucocorticoids induce increased expression of lipocortin by leukocytes (Perretti and Flower, 1996) and other cell types (Blackwell et al., 1982). Lipocortin also decreases neutrophil migration to inflammatory sites (Getting et al., 1997) which could be mediated by lipocortin-induced L-selectin shedding. Of note, lipocortin inhibits BK-induced plasma extravasation (Green et al., 1998). Further investigation will be required to determine which hormonal mediator(s) induces L-selectin shedding in this model.

Figure 27. Schematic diagram of circuitry mediating inhibition of synovial plasma extravasation induced by electrical stimulation.

Synovial plasma extravasation is inhibited when C fibers in the hind paw are electrically stimulated. This inhibition is dependent on both an intact spinal cord and HPA axis.

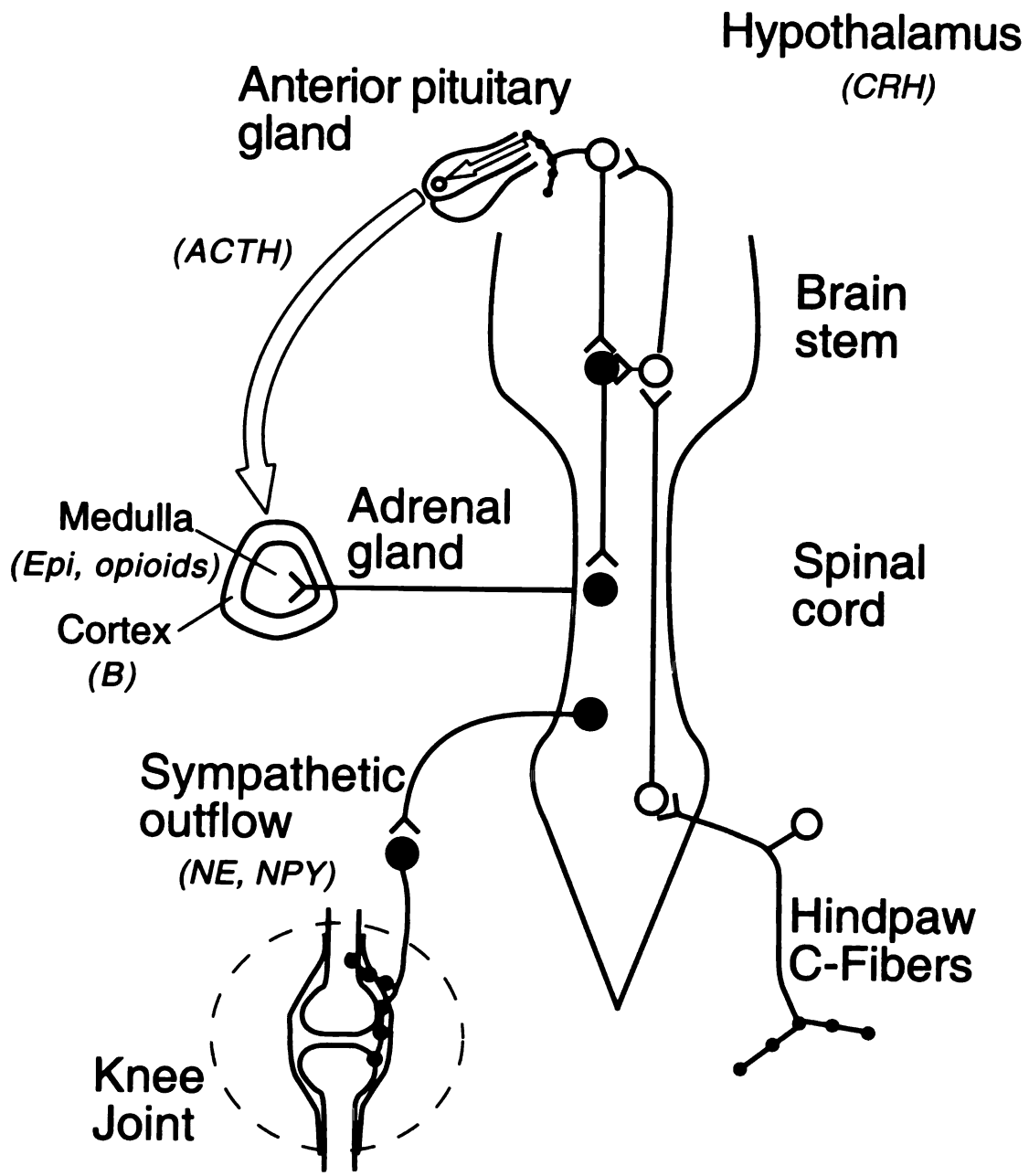

Hypothalamus
(CRH)
Anterior pituitary gland
(ACTH)
Brain stem
Medulla
(Epi, opioids)
Adrenal gland
Cortex
(B)
Spinal cord
Sympathetic outflow
(NE, NPY)
Hindpaw C-Fibers
Knee Joint

Figure 28. Noxious stimulation inhibits bradykinin-induced knee joint plasma extravasation.

BK induces sustained plasma extravasation (filled circles, n = 11). Noxious stimulation of the hindpaw inhibits BK-induced plasma extravasation (open diamonds, n = 12) while non-noxious stimulation does not (open triangles, n = 7).

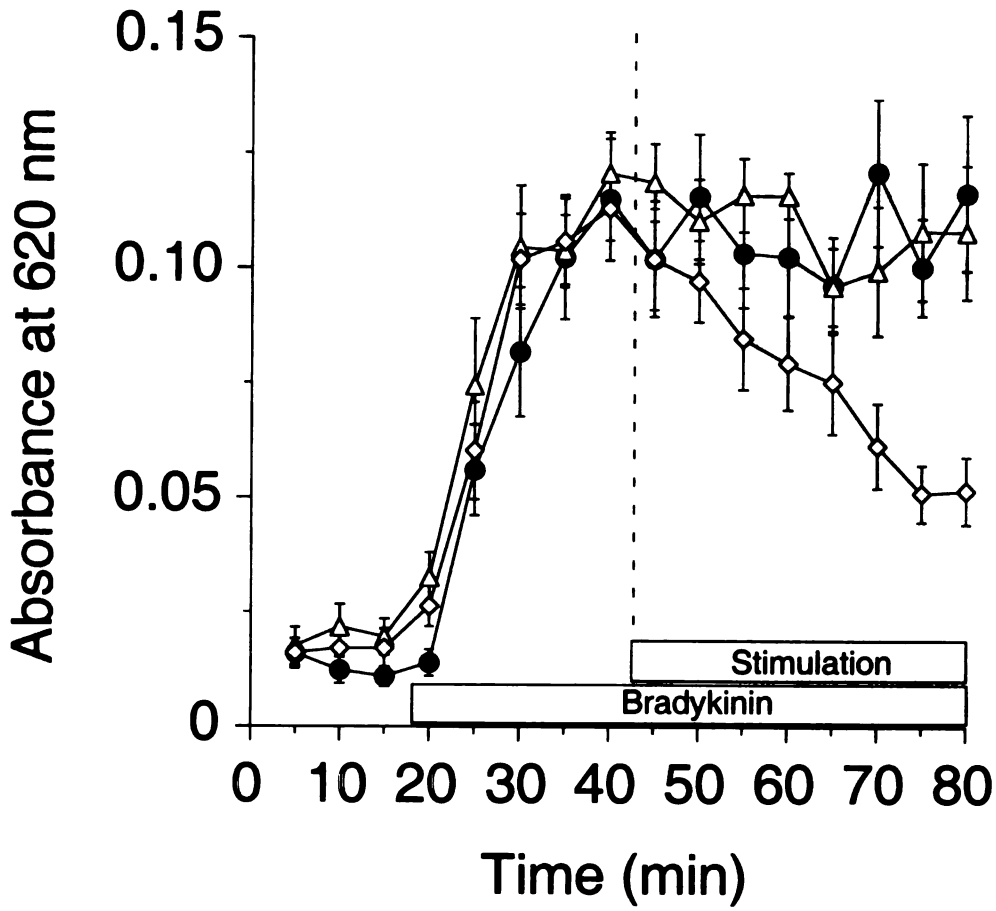

0.15
0.10
0.05
0
Absorbance at 620 nm
Stimulation
Bradykinin
0 10 20 30 40 50 60 70 80
Time (min)

Figure 29. Neutrophil depleting serum induces a rapid and sustained depletion of neutrophils.

Neutrophil depleting serum induced a pronounced inhibition of circulating neutrophils 20 and 40 minutes after administration of the serum (open circles, n=5). Neutrophil numbers began to recover 60 minutes after administration of the serum. In a separate group of rats (filled circles, n=5) neutrophil numbers were unaffected 20 and 40 minutes after administration of control pre-immune serum. These rats did, however, show a large increase in neutrophil numbers 60 minutes after administration of pre-immune serum.

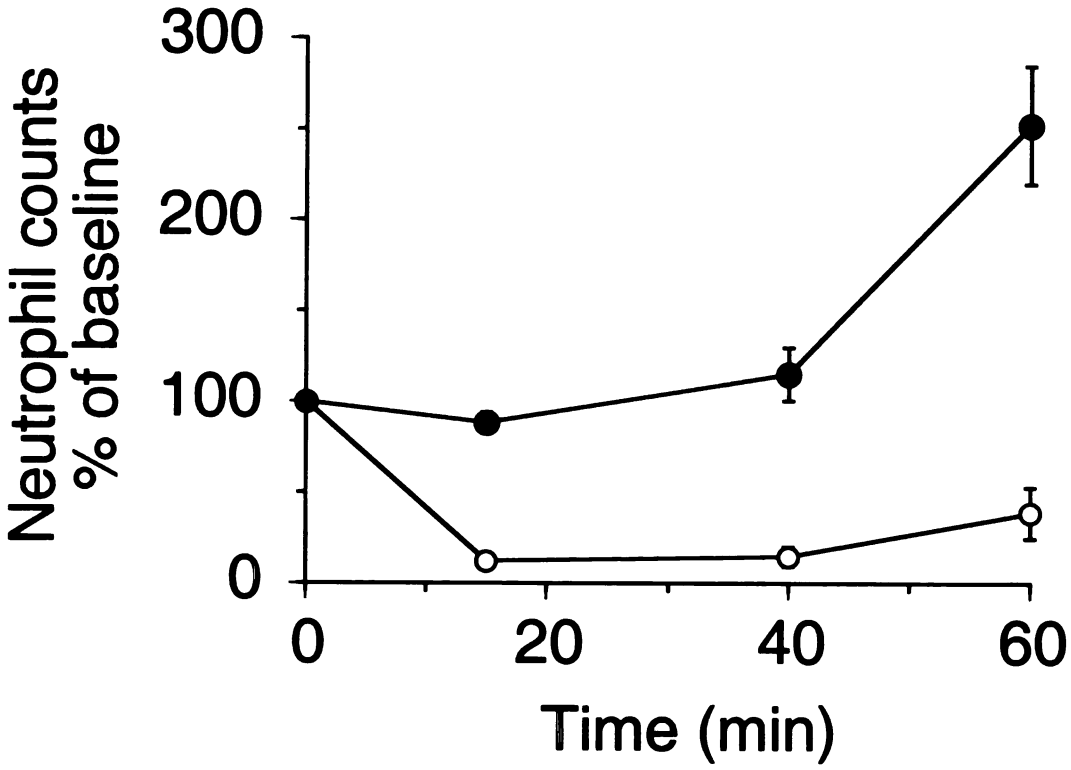

Neutrophil counts
% of baseline
300
200
100
0
0
20
40
60
Time (min)

Figure 30. Bradykinin-induced plasma extravasation is neutrophil dependent.

BK-induced plasma extravasation is significantly inhibited in rats pre-treated with neutrophil-depleting serum (open triangles, n = 7) compared to BK-induced plasma extravasation in rats pre-treated with pre-immune serum (filled circles, n = 7).

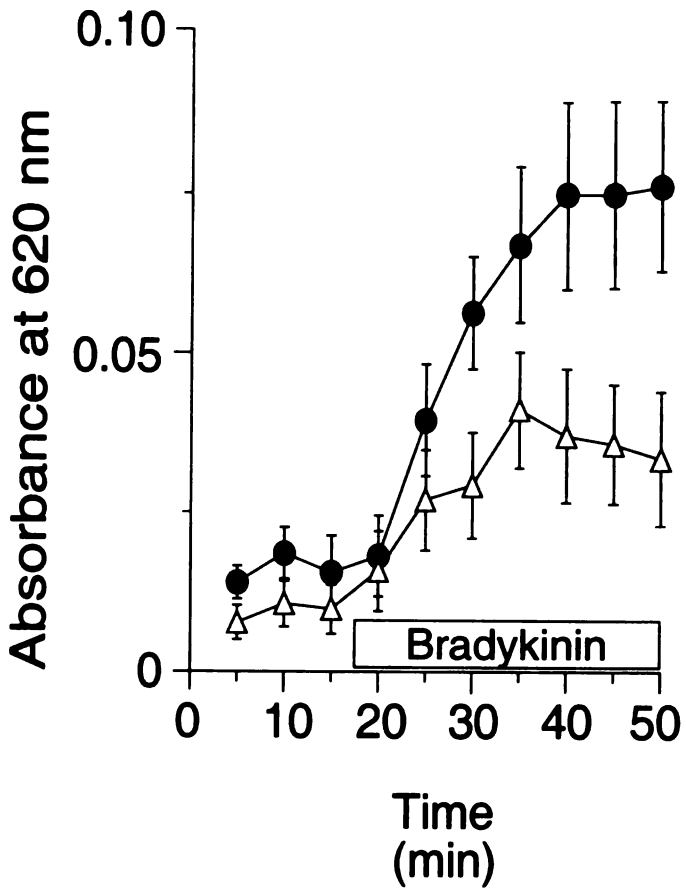

0.10
0.05
0
Absorbance at 620 nm
Bradykinin
0
10
20
30
40
50
Time
(min)

Figure 31. Noxious stimulation inhibits bradykinin-induced neutrophil recruitment.

A. Section through the synovium of a rat knee joint perfused with BK. B. Section through the synovium of a rat knee joint from a rat that received noxious stimulation in addition to BK perfusion through the knee joint. In A and B arrowheads denote post-capillary venules. In A small box denotes area magnified in inset. Scale bar is equal to 200 μm in A and B and is equal to 120 μm in inset.

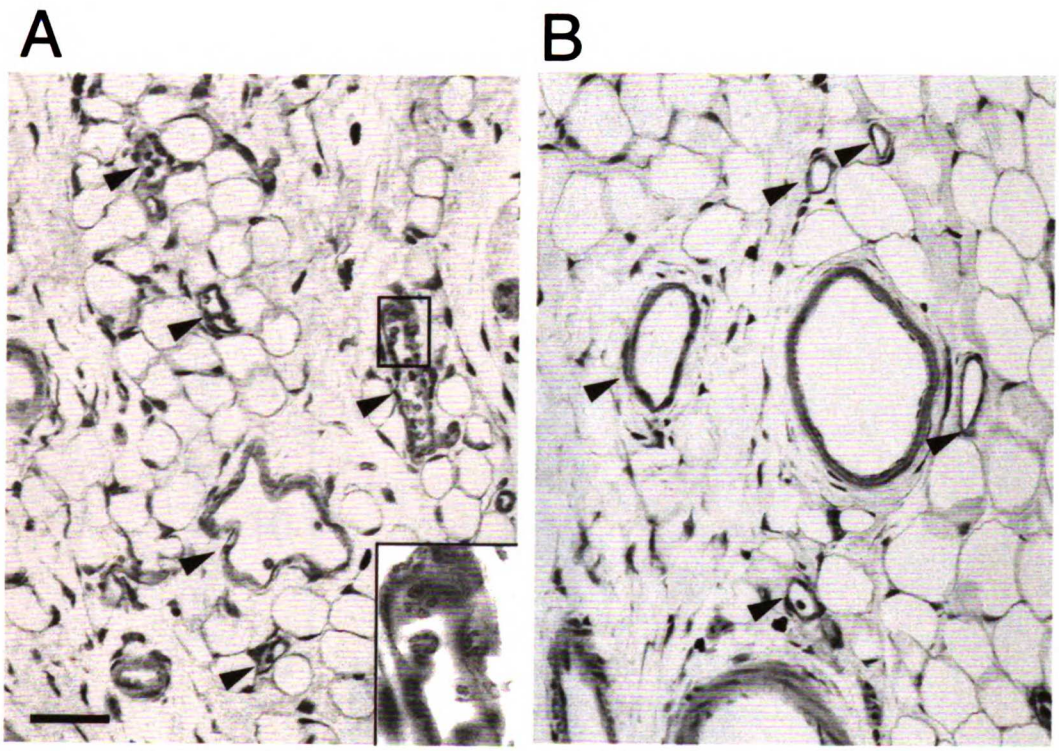

A
B

Figure 32. Quantification of noxious stimulation-induced inhibition of neutrophil recruitment.

Neutrophil numbers were quantified, in a blinded fashion, in rat knee joint synovia that had been perfused with saline (n=7) or BK (n=13) and in a third group of synovia (n=6) from rats that received noxious stimulation to the hindpaw during BK perfusion of the knee joint.

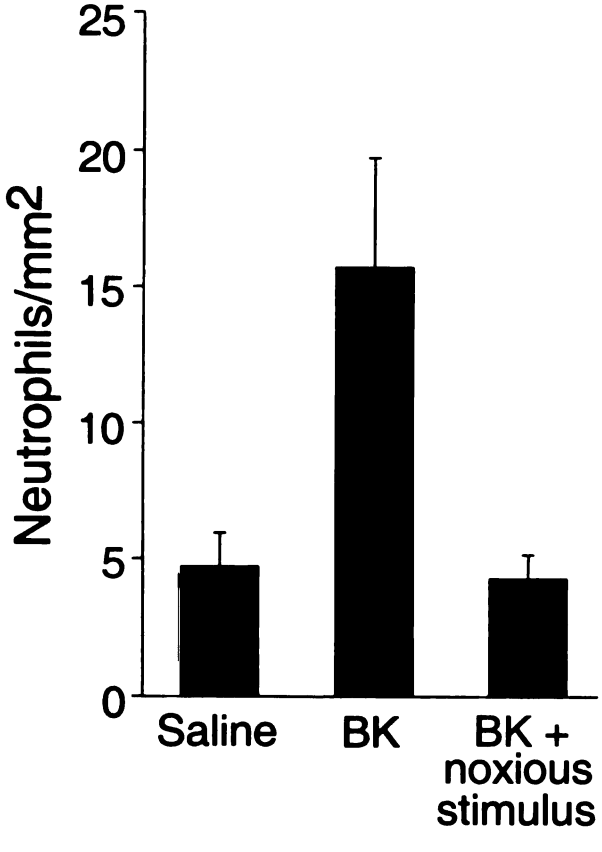

25
20
15
10
5
0
Neutrophils/mm2
Saline
BK
BK +
noxious
stimulus

Figure 33. Systemic block of L-selectin inhibits plasma extravasation.

Rats treated with an L-selectin blocking antibody (HRL-3, F (ab)′$_2$, 0.5 mg/kg intravenous; open triangles, n = 11) exhibit decreased BK-induced knee joint plasma extravasation while rats treated with an isotype-matched control antibody (hamster IgG, F (ab)′$_2$, 0.5 mg/kg, intravenous; filled circles, n = 10) do not.

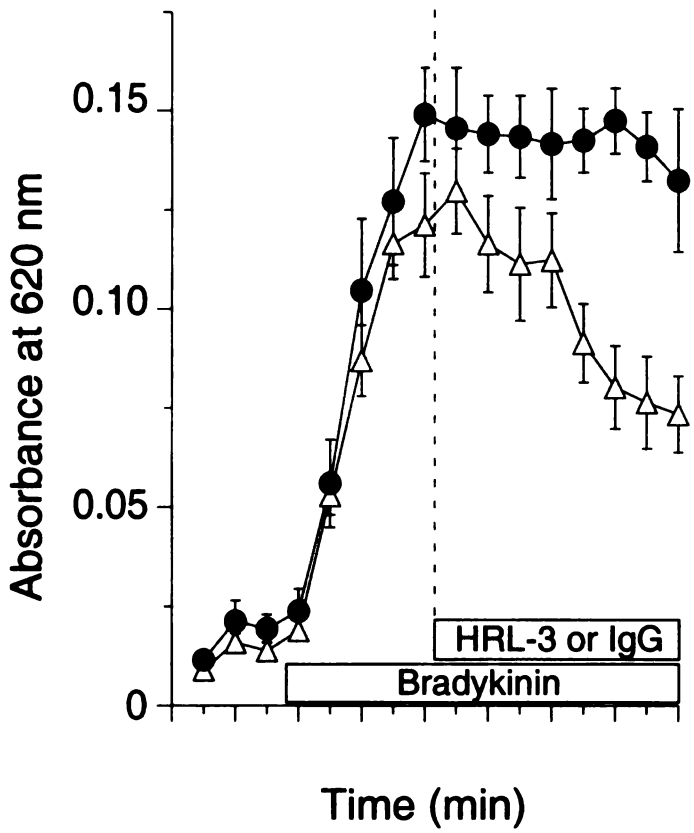

0.15
0.10
0.05
0
Absorbance at 620 nm
HRL-3 or IgG
Bradykinin
Time (min)

Figure 34. Noxious, but not non-noxious, stimulation induces L-selectin shedding from neutrophils.

Baseline L-selectin expression on neutrophils (upper panel, shaded area) was reduced to near background levels (upper panel, dashed line) in a substantial proportion of neutrophils 30 minutes after noxious stimulation (upper panel, solid line). In contrast, L-selectin expression on neutrophils was slightly increased after non-noxious stimulation (lower panel, solid line) compared to baseline (lower panel, shaded area). The dashed line (lower panel) indicates background staining.

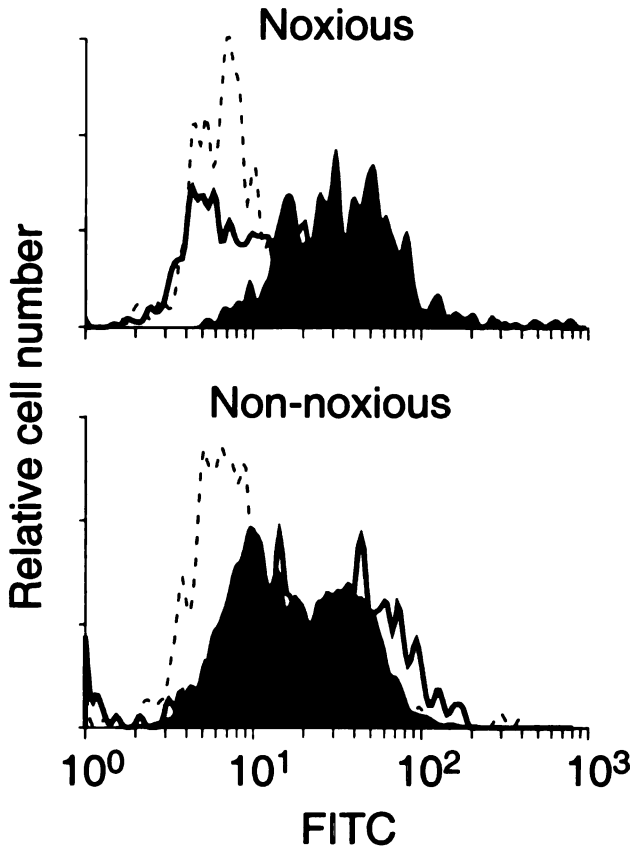

Noxious
Non-noxious
Relative cell number
10^0
10^1
10^2
10^3
FITC

Figure 35. Quantification of noxious stimulation-induced L-selectin shedding.

The average of several experiments indicates that L-selectin expression on neutrophils is significantly decreased after noxious (n = 5) but not after non-noxious stimulation (n = 6). In each case L-selectin on neutrophils collected after electrical stimulation was expressed as a percentage change from L-selectin expression prior to stimulation.

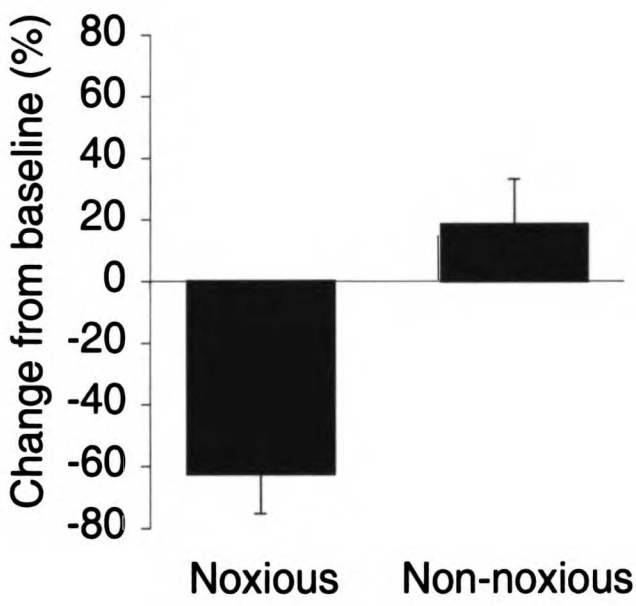

Change from baseline (%)
80
60
40
20
0
-20
-40
-60
-80
Noxious
Non-noxious

Figure 36. Noxious stimulation-induced L-selectin shedding results in decreased neutrophil tethering and rolling.

In vitro tethering and rolling of neutrophils isolated from rats that received noxious stimulation applied to the hindpaw (n = 9 from two preparations) were significantly reduced compared to that of neutrophils isolated from rats that had received a non-noxious stimulus (n = 8 from 2 preparations).

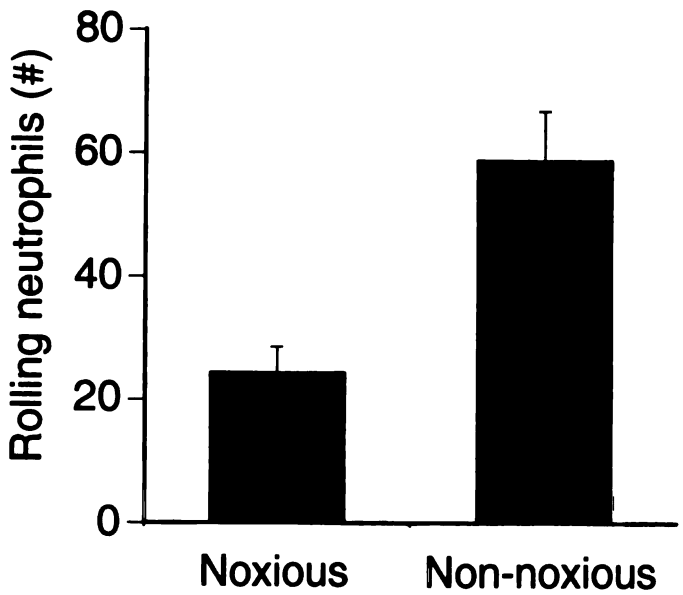

Rolling neutrophils (#)
80
60
40
20
0
Noxious
Non-noxious

Figure 37. Blocking L-selectin shedding blocks noxious stimulation-induced inhibition of plasma extravasation.

The effect of electrical stimulation of the hindpaw on BK-induced knee joint plasma extravasation was assessed in 3 groups of rats. One group (filled diamonds, n= 10) received the TACE inhibitor, Ro31-9790 (100 mg/kg, intraperitoneal), concurrent with BK. This group of rats, as well as a non-treated group of rats, (open diamonds, n = 12) then received a noxious stimulus to the hindpaw while a third group of rats (open triangles, n = 7) received non-noxious electrical stimulation. As shown previously, noxious, but not non-noxious, stimulation induced inhibition of plasma extravasation. However, Ro31-9790 blocked the inhibition of plasma extravasation induced by noxious stimulation of the hindpaw since BK-induced plasma extravasation in this group was not significantly different from that in the group that received non-noxious stimulation. Additionally, Ro31-9790 had no effect on BK-induced plasma extravasation prior to electrical stimulation since plasma extravasation was not significantly different in Ro31-9790-treated rats compared to untreated control rats, suggesting that Ro31-9790 does not potentiate plasma extravasation on-specifically in this model.

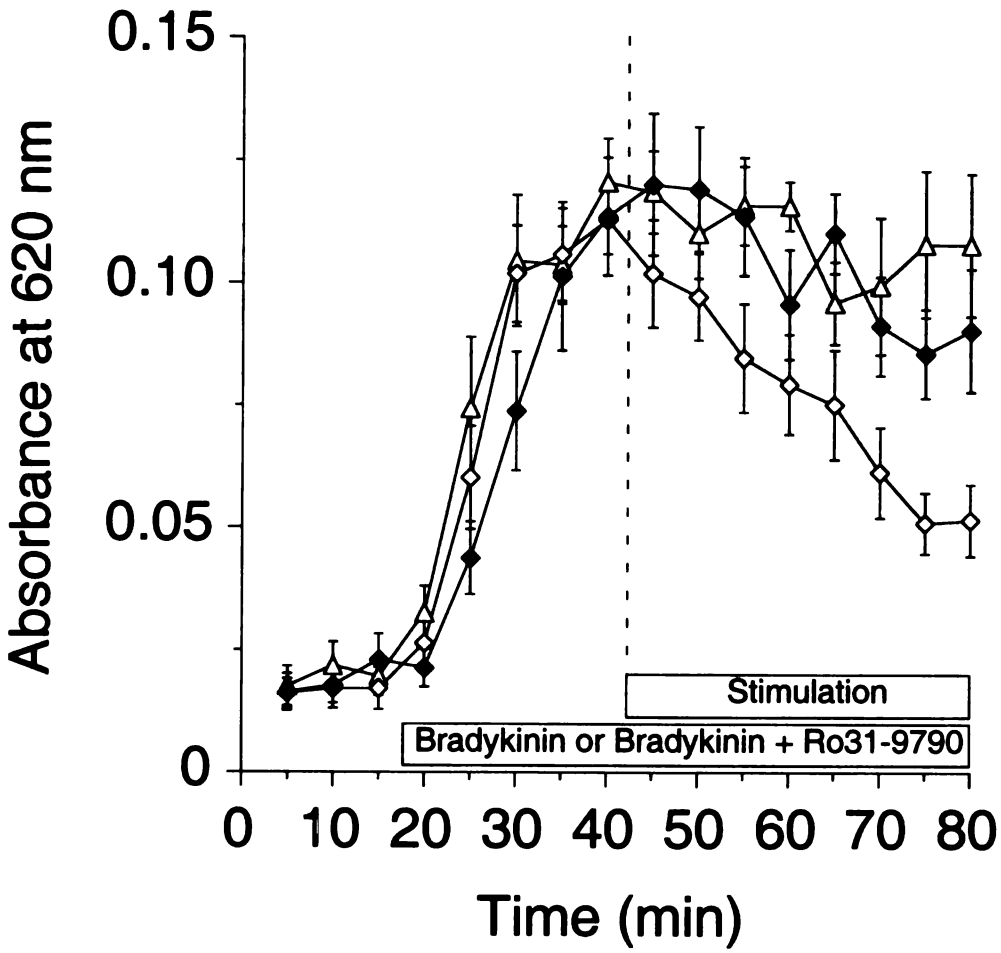

0.15
0.10
0.05
0
Absorbance at 620 nm
0 10 20 30 40 50 60 70 80
Time (min)
Stimulation
Bradykinin or Bradykinin + Ro31-9790

Table-1. Ro31-9790 blocks L-selectin shedding in nociceptor-stimulated rats.

Treatment	**% Change From Baseline** Mean (sem)
Noxious stimulation (n = 5)	-62 (12)
Noxious stimulation + Ro31-9790 (n = 4)	+25 (12)
Non-noxious stimulation (n = 6)	+18 (14)

CHAPTER SIX

GENERAL CONCLUSIONS

In these studies we have shown that stress exerts powerful inhibitory effects on one integral component of the inflammatory response, neurogenic plasma extravasation *in vivo*. In the first part of this investigation we showed that chronic, intermittent stress inhibits plasma extravasation while acute stress has no effect. We further showed that these effects are generalizable since three distinct stressors induced them. We next investigated the neuroendocrine mechanism of restraint stress-induced inhibition of plasma extravasation. We found that the HPA axis and specifically corticosterone mediate stress-induced inhibition of plasma extravasation. Corticosterone does not, however, inhibit plasma extravasation in a simple, direct way since repeated pulses of corticosterone are required to induce inhibition. Finally, we explored the local cellular mechanisms of stress-induced inhibition of plasma extravasation. We found that stress-induced inhibition of plasma extravasation is mediated by an effect on neutrophils. Specifically, the stressful stimulus nearly abolished BK-induced neutrophil accumulation, an event necessary for induction of plasma extravasation, by inducing shedding of L-selectin from circulating neutrophils.

The overall goal of this body of work was to use *in vivo* approaches to contribute information to the ultimate understanding of the relationship between stress and disease. We chose to investigate inflammation since an inappropriate inflammatory response is a central feature of many diseases (e.g., rheumatoid arthritis, colitis and asthma). Other investigators, using similar *in vivo* approaches, have found that stress inhibits other complex immune responses. For example, stress decreases the ability of animals to eradicate HSV-1 virus (Brenner and Moynihan, 1997; Sheridan and Dobbs, 1994), inhibits antibody production directed to specific antigens (Glaser et

al., 1998; Moynihan et al., 1994), and increases time of wound healing (Padgett et al., 1998). Our data indicating that stress inhibits plasma extravasation is consistent with this literature showing stress-induced inhibition of *in vivo* immune responses.

What might these findings suggest about the relationship between stress and disease? The findings that stress decreases viral clearance (Brenner and Moynihan, 1997; Sheridan and Dobbs, 1994) and antibody production (Glaser et al., 1998; Moynihan et al., 1994) suggest that stress could contribute to increased morbidity of diseases that have infectious etiologies. Understanding the theoretical impact of our findings on disease is more complex. Central to chronic inflammatory disease is an uncontrolled inflammatory response. This uncontrolled inflammatory response is generally the target of treatment and agents are administered to dampen the response. Do our findings that stress inhibits a component of the inflammatory response suggest then that stress should be beneficial for patients with inflammatory disease? The literature suggests not, as human studies have drawn positive correlations between inflammatory disease activity and psychological stress. For example, patients undergoing high levels of mild daily stress show exacerbation of signs and symptoms of rheumatoid arthritis (Potter and Zautra, 1997; Zautra et al., 1994), ulcerative colitis (Levenstein et al., 1994) and inflammatory bowel disease (Dancey et al., 1998). Additionally, higher levels of perceived stress are associated with increased incidence of asthmatic episodes (Sekas and Wile, 1980). Both patients with rheumatoid arthritis (Affleck et al., 1987) and inflammatory bowel disease (Robertson et al., 1989) cite stress as the most frequent cause of symptom flares and stress management

interventions can reduce these symptom flares in rheumatoid arthritis patients (Parker et al., 1995).

How can the finding that stress inhibits a component of the inflammatory response be reconciled with findings from studies of patients with inflammatory disease that indicate that stress exacerbates inflammatory disease activity? It is clear that the inflammatory response in persons with inflammatory disease is not normal. It is possible that in this altered state some components of the inflammatory response would ameliorate disease activity while other components would exacerbate it. In fact inhibition of plasma extravasation has been associated with worsening of radiological joint injury in experimental arthritis (Coderre et al., 1991; Green et al., 1991; Miao et al., 1992) and as experimental arthritis progresses inducible plasma extravasation decreases (Miao et al., 1992). Plasma extravasation may exert this protective effect by increasing extravasation of plasma proteinase inhibitors (e.g., α_1-proteinase inhibitor, α_1-anti-chymotrypsin and α_2-macroglobulin) that control excessive proteolytic activity and thereby protect against connective tissue damage (Kozik et al., 1998). Additionally, increased plasma extravasation may increase the rate of removal of tissue-injurious products of the inflammatory response. If plasma extravasation is protective in the setting of inflammatory disease, then inhibition of it by stress would be expected to exacerbate inflammatory disease making the current findings consistent with patient reports regarding the impact of stress on their disease. Clearly more investigation is required to understand the implications of stress-induced inhibition of plasma extravasation for inflammatory disease.

The study of the relationship between stress and inflammatory diseases is in its infancy. Ultimately, an understanding of the relationship between stress and inflammatory disease and the ability to manipulate that relationship for better health will come from a synthesis of many investigations that focus on key variables. These variables include all components of the inflammatory response (i.e., plasma extravasation, leukocyte recruitment, blood flow and pain) and of the nature of the stressor (i.e., intensity of the stressor, duration of the stressor and timing of the stressor relative to disease onset).

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