Serum calcitonin gene-related peptide (CGRP) concentrations in the horse and their relationship to the systemic inflammatory response

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(ABSTRACT)

Systemic inflammation is a leading cause of mortality and morbidity in both human and equine intensive care patients. This systemic inflammatory response may be due to insult from bacterial, viral, fungal or parasitic invasion or from trauma or hypoxemia. Local and systemic release of a wide variety of endogenous pro-inflammatory mediators results in activation of the innate immune system in order to resolve the insult. In sepsis this initial appropriate host response becomes amplified and deregulated leading to refractory hypotension and multiple organ dysfunction. The exact incidence of sepsis (SIRS due to bacterial infection) has not been reported in the equine literature (Roy 2004). Since early recognition and treatment of sepsis are associated with improved outcome the search for markers to accurately predict presence of sepsis and likelihood of survival continues. The serum concentration of both procalcitonin and its related molecule CGRP have been documented to increase in humans with SIRS, yet no literature exists as to the production or role of CGRP in equine patients with SIRS.

This study showed that equine CGRP was produced in detectable quantities by healthy adult horses and neonatal foals less than two weeks of age using a rat α-CGRP ELISA. The low percentage recovery of CGRP from samples and the high lower limit of detection for the assay prevented establishment of a normal concentration range of CGRP in healthy horses. In both adult horses and foals with documented SIRS, CGRP concentrations were significantly increased at time of presentation to the hospital ($p<0.0002$, $p<0.003$ respectively). A trend towards increased serum CGRP concentration was present in anesethized horses exposed to endotoxin, but this was not statistically significant ($p< 0.067$).

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List of abbreviations

α	alpha
AchE	acetylcholinesterase
AMP	adenosine monophosphate
ADM	adrenomedullin
APACHE	acute physiology and chronic health evaluation
β	beta
BP	blood pressure
CALC	calcitonin gene
CARS	compensatory anti-inflammatory response
CD	cluster of differentiation
CLP	cecal ligation and puncture
CGRP	calcitonin gene related peptide
CRSP	calcitonin receptor stimulating peptide
CRLR	calcitonin receptor like receptor
CSF	cerebrospinal fluid
CT	calcitonin
CV	coefficient of variance
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
Dr	doctor
DRG	dorsal root ganglion
E2	estradiol
E.coli	*Escherichia coli*
ELISA	enzyme linked immunoassay
Fab	fluorescent antibody
g	gram
GMP	guanosine monophosphate
h	hour
i-CGRP	CGRP imunoreactive nerve fibers
ICU	intensive care unit

IL1	interleukin 1
kPa	kilopascals
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LTA	lipotechoic acid
M	molar
MHC	major histocompatability complex
mm	millimeter
mmHg	millimeters of mercury
MODS	multiple organ dysfunction
MOFS	multiple organ failure
mRNA	messenger ribonucleic acid
NE	norepinepherine
NEB	neuroepithelial body
NEP	neuroendopeptidase
NGF	nerve growth factor
NO	nitric oxide
NOS	nitric oxide synthase
NSB	non-specific binding
O_2	oxygen
p	pico
P4	progesterone
$PaCO_2$	arterial partial pressure of carbon dioxide
PaO_2	arterial partial pressure of oxygen
PG	prostaglandin
PKA	phosphokinase
PNEC	pulmonary neuroendocrine cells
RAAS	renin-angiotensin-aldosterone system
RAMP	receptor activity modifying protein
RCP	receptor component protein
RIA	radioimmunoassay

SEM	standard error of the mean
SHR	spontaneously hypertensive rats
SIRS	systemic inflammatory response syndrome
SP	substance P
SVR	systemic vascular resistance
TDI	toludine di-isocyanate
TLR	toll like receptor
TNF-α	tumor necrosis factor alpha
VIP	vasoactive intestinal peptide
WBC	white blood cell

Introduction

Calcitonin gene-related peptide and the systemic inflammatory response syndrome.

Systemic inflammation is a leading cause of mortality and morbidity in both human and equine intensive care patients. This systemic inflammatory response may be due to insult from bacterial, viral, fungal or parasitic invasion or from trauma or hypoxemia. Sepsis has been defined as the systemic inflammatory response to infection. The inciting focus of sepsis, via either exotoxins or microbial structural components, causes local and systemic release of a wide variety of endogenous inflammatory mediators, such as tumor necrosis factor alpha, interleukin 1, platelet activating factor, oxygen free radicals and arachadonic acid metabolites. This initial appropriate host response becomes amplified and deregulated leading to refractory hypotension, multiple organ dysfunction and high patient morbidity and mortality. Two distinct peptides of the calcitonin I gene, procalcitonin and CGRP have been found to be elevated in human patients with sepsis, and CGRP has a physiological role in maintenance of vasomotor tone. Since abnormalities in cardiovascular tone are of paramount importance in the development of sepsis and septic shock, much work has been focused on the role of CGRP in the pathophysiology of human sepsis, both as an instigator of the abnormalities in vascular tone and as a possible marker in predicting sepsis or mortality associated with sepsis (Beer *et al.* 2002). To date, no literature exists as to CGRP concentrations in the horse, either in health or disease.

Chapter 1 calcitonin gene-related peptide in health and disease.

Introduction

CGRP, located primarily in capsaicin sensitive sensory neurons on the peripheral and central nervous system has important roles in the physiological function and homeostatic maintenance of the cardiovascular, respiratory, gastrointestinal, integumentary, neurological, and reproductive systems of mammalian species.

Molecular Genetics of calcitonin gene related peptide (CGRP)

The calcitonin/calcitonin gene related peptide (CGRP) gene complex encodes a small family of peptides: calcitonin, CGRP, adrenomedullin and amylin. The calcitonin/ CGRP gene complex comprises 5 genes. In man, these genes are located on the short arm of chromosome 11, between the catalase and parathyroid hormone genes (Breimer *et al.* 1988). The calcitonin I gene (CALC I) gives rise to two distinct peptides, calcitonin (CT) and CGRP, by tissue specific alternative splicing of the pre-mRNA (Linscheid *et al.* 2005). The CALC I consists of six exons: the first three exons are present in both calcitonin and CGRP mRNA, though exon I is not translated. Exon IV contains the calcitonin coding sequence. Exon V encodes the CGRP sequence. The sixth exon, which is also part of the CGRP transcript, is not translated (Breimer *et al.* 1988). It has been shown that cells synthesizing calcitonin splice together exons I-IV to form mature mRNA, whereas cells producing CGRP splice together exons I-III, V and VI (Rosenfeld *et al.* 1981). The peptides are synthesized as large precursor molecules that are cleaved intracellularly to release the active molecule (Breimer *et al.* 1988) The first 25 amino acids constitute the leader sequence that determines secretion (Breimer *et al.* 1988).

The organization of the CALC II gene is similar to that of the CALC I. Exons III and V are similar, but exon VI is only somewhat alike (65%) (Breimer *et al.* 1988) and exon IV contains a stop codon (Linscheid *et al.* 2005). CALC I therefore produces CGRP-1 (α-CGRP) or calcitonin; CALC II produces CGRP II (β-CGRP) but not

calcitonin. Using calcitonin and CGRP-specific cDNA probes it has been shown that the alternative production of calcitonin or CGRP is regulated tissue specifically (Amara *et al.* 1982). Calcitonin III is a pseudogene. Adrenomedullin (ADM) is encoded by CALC IV and amylin by the CALC V gene (Linscheid *et al.* 2005).

Molecular Structure of CGRP

The CGRP protein consists of 37 amino acids with a disulphide bridge between positions 1 and 7 and a C-terminal phenylalanine residue. Human α CGRP differs from the predicted rat sequence by four amino acid substitutions (positions 1,3,25 and 35; three of these involve charge changes). Human α CGRP differs from the β form by three amino acids (positions 3,22 and 25). Two of these three residues (positions 3 and 25) have also been modified in rat α CGRP: one of these is replaced by the same amino acid (asparginine) (Breimer *et al.* 1988). Very recently, calcitonin receptor stimulating peptide (CRSP), a new member of the calcitonin gene related peptide family has been identified. The peptide properties indicate that CRSP probably elicits its biological effects via the calcitonin receptor (Springer *et al.* 2003)

Toribio et al. first published the nucleotide and amino acid sequences of CT and CGRP in the horse (Toribio *et al.* 2003). An equine thyroid gland cDNA library was created containing $1.2x10^6$ cloned recombinants. These were screened with ovine CT probes that had 80% homology with equine CT and CGRP-I and 90% homology with equine CGRP-II (Toribio *et al.* 2003) and were radiolabelled with ($^{\alpha\text{-}32}$P) dATP. Screening revealed four clones (A1, A4, B1 and B2) that hybridized to the CT/CGRP probe and contained complete cDNA sequences for CT and CGRP. The cDNA sequences for the signal and N-terminal peptides of clone B1 were identical to A4, indicating that these clones came from the same gene. The product was called equine CGRP-I. The amino acid sequence for the signal and N-terminal peptides of clone A1 had 65% homology with clones A4 and B1. Clone A1 cDNA likely represents a gene equivalent to CALC-II, and the peptide encoded for by clone A1 was named CGRP-II. The degree of homology of mature equine CGRP-I to CGRP in other species is low (<59%). The degree

of homology between equine CGRP-I and equine CGRP-II was also low (56%), compared to 91% between human CGRP-I and human CGRP-II (Toribio *et al.* 2003) (see figure 1). However equine CGRP-II has higher homologies (>80%) to CGRP-I's of chicken, rat, ovine, swine and bovine species, and the authors proposed that CGRP-II in horses may have assumed many of the functions attributed to CGRP-I in other species (Toribio *et al.* 2003).

Storage and Release of CGRP

CGRP is co-stored and co-localized with substance P (SP) in afferent capsaicin sensitive sensory nerves. As with many neuropeptides in primary afferent neurons, CGRP can be released by peripheral noxious electrical and chemical stimulation. Electrical stimulation of sensory nerves, especially the vagus, trigeminal, and saphenous nerves in the guinea pig and rat have been used to study the release of neuropeptides. Also receptors for inflammatory mediators such as histamine and bradykinin are present on sensory nerves. They may further increase the release of neuropeptides such as CGRP (Springer *et al.* 2003). In the condition of inflammation, as found is asthma, other locally produced substances such as nitric oxide, or leukotrienes also have the potential to modulate CGRP release (Eynott *et al.* 2002; Holgate *et al.* 2003) In the vagal sensory system, CGRP is synthesized nearly exclusively in the small-diameter nociceptive type C-fiber neurons, which respond to noxious or tissue damaging stimuli. CGRP mRNA as well as mature peptide have also been detected in pulmonary neuroendocrine cells (PNEC) of both the solitary type and cluster type (neuroepithelial body: NEB) at all levels of the airway epithelium from bronchi and alveoli (Springer *et al.* 2003).

Capsaicin sensitive nerves have the capsaicin or vanilloid receptor subtype 1 (VR1), which is a non-selective cation channel and can be activated by vallinoids, heat stimulus and hydrogen ions. Activation of VR1 causes the release of CGRP, SP and other tachykinins (Deng and Li 2005). It has been reported that CGRP can be released from capsaicin-sensitive sensory neurons in a spillover manner in the absence of afferent nerve stimulation, implying that these neurons participate in the continuous regulation of

vascular tone (Wimalawansa 1996). Capsaicin-sensitive sensory nerve terminals can release CGRP in response to local factors including nerve growth factor (NGF), vascular wall tension, bradykinin/prostaglandins, endothelin and the sympathetic nervous system (Deng and Li 2005). It is possible that the factors altering acute release of CGRP can also modulate the long term production and release of this peptide. Studies using primary cultures of adult rat dorsa root ganglion (DRG) have shown that NGF, bradykinin/prostaglandins, protein kinase A activator and protein kinase C activator can stimulate CGRP synthesis and release (Deng and Li 2005).

Estrogen or progesterone per se have no direct effect of CGRP synthesis and release in the cultures of adult rat DRG. However these two female sex steroid hormones increase the synthesis and release of CGRP in DRG neurons in the presence of NGF in vitro (Deng and Li 2005). Reversely dexamethasone can inhibit the expression of CGRP in the DRG neurons in vitro, which is mediated by attenuation of the stimulatory affects of NGF. Several different classes of regulatory factors act to modulate CGRP synthesis and release by antagonizing or activating NGF-stimulated expression and release of CGRP (Deng and Li 2005). A possible mechanism of NGF induced production of CGRP has been suggested. Studies in primary cultures of sensory neurons have recently addressed the mechanisms controlling the CGRP promoter region (Durham and Russo 2003). Using reporter gene assays and transient transfections, it was shown that an 18bp enhancer containing a helix-loop-helix element is necessary for promoter activity and subsequent gene transcription.

Distribution of CGRP containing neurons

CGRP containing neurons are widely distributed in the nervous and cardiovascular systems. In the peripheral nervous system, the prominent site of CGRP synthesis is the DRG, which contains the cell bodies of capsaicin-sensitive sensory neurons. The peripheral processes of these sensory neurons terminate on blood vessels (Deng and Li 2005). Their central processes terminate in laminae I and II of the dorsal horn and also synapse with the intermediolateral nucleus of the spinal cord, which

contains the sympathetic pre-ganglionic neurons. This connection could influence the activity of the sympathetic nervous system, and thus vascular tone and blood pressure (BP). Most blood vessels are surrounded by a dense perivascular CGRPergic neural network, which is found at the junction of the adventia and the media, passing into the muscle layer (Wimalawansa 1996). CGRP is released from these CGRPergic sensory neurons and pulmonary neuroendocrine cells in response to both inflammatory mediators and noxious stimuli. CRRP may act locally as a paracrine molecule or at more distant site leading to measurable quantities in the systemic circulation

In the cardiovascular system the highest density of CGRP binding sites are present in the heart and blood vessels (Arulmani *et al.* 2004). In the heart, high affinity binding sites for CGRP are found in both atrial and ventricular preparations (Arulmani *et al.* 2004). Regardless of the species, the density of the CGRP binding sites in atria invariably exceeds that of ventricles (Chang *et al.* 2001). Autoradiographic studies in the heart of rats, guinea pigs and humans (Arulmani *et al.* 2004), have shown the highest density of CGRP binding sites in the coronary arteries, coronary veins and in the heart valves, while a lower number is found in the coronary arterioles and endocardium (Arulmani *et al.* 2004). CRGP receptors are also abundantly present in the thyroid gland, gastrointestinal tract, parathyroid gland, adrenals, pituitary, exocrine pancreas, kidneys, uterus, bones, skin and skeletal muscles (Wimalawansa 1996). In adult horses expression of CGRP-I and II has been found in the pituitary gland, kidney, lung, liver and prostate gland. (Toribio *et al.* 2003)

Degradation of CGRP

CGRP may be subject to degradation by several enzymes including neuroendopeptidase (NEP), tryptase and chymotrypsin (Deng and Li 2005). The sequence of rat alpha-CGRP contains the tetrapeptide eosinophil granulocyte chemotactic factor Val32-Gly-Ser-Glu35. This tetrapeptide fragment was generated following cleavage at a substrate recognition site unusual for NEP and the chemotactic activity of the rat alpha-CGRP was increased after hydrolysis. In contrast rat beta-CGRP, which

lacks the tetrapetide sequence is completely devoid of chemotactic activity. Val-Gly-Ser-Glu was identified as the principal fragment with chemotactic activity towards eosinophilic polymorphonuclear leukocytes (Davies *et al.* 1992). The half life of CGRP in mammalian plasma is approximately 10 minutes (Deng and Li 2005).

Mechanism of action of CGRP

CGRP acts on G-protein coupled receptors that are coupled via Gs to adenylate cyclase, leading to an induction of intracellular cyclic AMP formation. A second pathway of signal transduction is based on the stimulation of phospholipase C-beta 1 which requires the pertussis toxin insensitive G alpha q/11 subunit to release Ca2+ via the inositol 1,4,5-triphosphate pathway (Drissi *et al.* 1998). CGRP receptors, along with other receptors for neuropeptides such as vasoactive intestinal peptide (VIP), belong to the family of seven transmembrane receptors and that their activity depends upon receptor activity-modifying proteins (RAMPS). The first identified human CGRP receptor contains 461 amino acids and forms a 7 transmembrane spanning motif consisting of 16 to 28 hydrophobic amino acids each. CGRP-A and CGRP-B receptor subtypes have been suggested with regard to the differential action of the agonist [Cys(ACM2,7)]CGRP ($CGRP_2$-selective) and the antagonist CGRP-8-37($CGRP_1$-selective, pK_i 7.5-8.0) (Springer *et al.* 2003). A member of a single transmembrane domain protein family determines the specificity for CGRP (RAMP1) or the related adrenomedullin (RAMP2) (Springer *et al.* 2003). Recent observations have demonstrated that a family of RAMPS modulates the activity of receptors for CGRP and its related peptides (McLatchie *et al.* 1998). Thee RAMPs appear to function as chaperones for CGRP receptors and are required for their function. Cotranfection of the CL-receptor (historically calcitonin receptor-like receptor) with $RAMP_1$ leads to the expression of high affinity receptors for CGRP (CGRP-A). In contrast, the cotransfection of the CL-receptor with $RAMP_2$ results in the expression of high affinity receptors for adrenomedullin (McLatchie *et al.* 1998). An accessory protein termed receptor component protein (RCP) also influences CRGP binding and function, and next to CGRP-A receptor, a new receptor termed CGRP-B may exist.

Physiological Actions of CGRP

Vascular effects

Nerve fibers that contain CGRP have been identified in the brain, spinal cord and in association with blood vessels in most peripheral organs (Yallampalli *et al.* 2002). CGRP is a potent vasodilator, approximately 100-1000 times more potent than other vasodilators such as adenosine, SP or acetylcholine (Brain *et al.* 1985), and it possesses positive chronotropic and inotropic effects (Asimakis *et al.* 1987). Several lines of evidence suggest that CGRP plays an important role in the regulation of vascular resistance and regional organ blood flow, both under normal physiological conditions and in the pathology of hypertension in man. Intracerebroventricular administration of CGRP evokes a transient elevation in mean arterial pressure and sustained tachycardia in rats (Deng and Li 2005). In contrast, systemic administration of CGRP decreases blood pressure in a dose dependent manner, coupled with a more sustained tachycardia in normotensive animals and humans (Wimalawansa 1996). The primary mechanism responsible for the depressor effect is peripheral arteriolar dilation which consists of (1) reducing intracellular Ca2+ with inhibition of Ca2+ influx and Ca2+ release from sarcoplasmic reticulum; (2) reducing the sensitivity of contractile unit response to Ca2+; (3) increasing intracellular cyclic adenosine monophosphate (cAMP); (4) activating ATP-activated potassium (K+) channels; and (5) releasing nitric oxide (NO) (Wimalawansa 1996)' (Bell and McDermott 1996). The vasodilator response mediated by NO is endothelium dependent, while the other factors directly affect the vascular smooth muscle. The mechanism by which CGRP dilates blood vessels through endothelium dependent and/or independent manners are varied in different vascular beds (Bell and McDermott 1996). Vasodilatation is one of the principal effects of CGRP on the pulmonary and systemic vasculature. CGRP effectively dilates human pulmonary vessels in vitro. Bronchial vessels are also dilated by CGRP in vivo with a prolonged increase in airway blood flow in anaesthetized dogs (Salonen *et al.* 1988) and conscious sheep (Parsons *et al.* 1992). A recent study focused on the vascular effects of specific N-

terminal rat α-CGRP fragments containing the disulphide bridge (Cys(2)-Cys(7) with aminated C-terminal in chronic hypoxic pulmonary hypertension in rats. It was shown that the N-terminal CGRP fragments CGRP(1-8),CGRP(1-13) and CGRP(1-14) may have a protective role in hypoxic pulmonary hypertension as chronic infusion of the fragments at 7 nmol/hr/rat via the right jugular vein during 14 days of hypobaric hypoxia (10% inspired O2) significantly decreased the mean pulmonary artery pressure, right ventricular hypertrophy and pulmonary artery medial thickness in comparison with controls, while systemic pressure was unchanged (Qing *et al.* 2003).

Airway effects

CGRP has also been shown to be a constrictor of human bronchus airway smooth muscle in vitro, which is surprising since it stimulates intracellular cAMP generation. It was shown that CGRP causes a concentration dependent contraction of human bronchi (EC_{50} $4.9x10^{-9}$M) and that it is significantly more potent than SP or carbacol (Springer *et al.* 2003). The contractile responses were unaffected by atropine (2x10-6M), propanolol (10-6M), tetrodotoxin (3x10-6M), or indomethacin (4.9x10-9M), indicating the direct effect of CGRP on airway smooth muscle. There are two possible mechanisms which may account for this constrictory effect: Either CGRP activates phospholipase C-$beta_1$ and leads via the inositol 1,4,5-triphosphate-related pathway to constriction, or CGRP does not have a direct effect on the smooth muscle itself but liberates other constrictor mediators (Springer *et al.* 2003). In this respect, only a few binding sites for CGRP were found on smooth muscle areas in human lung, suggesting an indirect mode of CGRP mediated bronchoconstriction in human airways. In the guinea-pig respiratory tract the effects of CGRP on airway smooth muscle are controversial and no consistent bronchoconstrictory or bronchodilatory effect has been demonstrated. The effect on airway smooth muscle might result from the balance between mediators that cause functional opposite effects (Springer *et al.* 2003). Mucus secretory cells display only a low density of CGRP binding sites as shown by autoradiographic studies (Springer *et al.* 2003). However, CGRP may increase airway mucus secretion indirectly by the increase in blood flow to submucosal glands.

CGRP exerts a moderate inhibition of mucus secretion in ferret trachea induced by cholinergic stimulation (Springer *et al.* 2003) Here it was demonstrated that CGRP (1-100nM) produced a small concentration-dependent increase in basal mucus volume , lysozyme and albumin outputs. CGRP also caused a concentration-dependent inhibition of SP and methacholine-induced lysozyme output, but a concentration dependent increase in SP and methacholine induced albumin output. It was concluded that CGRP weakly stimulates basal secretion and epithelial albumin transport without altering epithelial cell integrity (Webber *et al.* 1991). A marked species-dependent variability is observed in the effects produced by CGRP in the secretion from seromucus glands. In fact an effect opposite (increased secretion) to that found in guinea pigs was observed in feline submucosal glands, where it was shown that that CGRP stimulates glycoconjugate secretion from airway submucosal glands by inducing Ca2+ influx from the extracellular solution (Springer *et al.* 2003). No studies nave been conducted on airway mucus secretion in human lower respiratory tracts (Springer *et al.* 2003). In addition to its role in regulation of vascular tone and bronchial smooth muscle tone, CGRP has a wide range of actions in physiological homeostasis.

Gastrointestinal effects

CGRP receptors are present on non-parenchyma hepatocytes suggesting a paracrine system could be involved in liver metabolism. CGRP is also synthesized by primary cultures of hepatocytes and in liver (Bracq *et al.* 1994). CGRP has also been shown to induce gall bladder relaxation via inhibition of cholecystokinin- induced constriction (Kline and Pang 1994) in iguanas. CGRP immunoreactivity is also present on pancreatic insulin producing cells in rats (Edwin and Leigh 1999), suggesting CGRP to be an endocrine peptide in addition to its neurological role. CGRP is also widely distributed in the enteric nervous system and gut afferents. However its role in normal digestion and absorption is not characterized. (Barada *et al.* 2000) In in-vivo experiments CGRP has been shown to reduce alanine absorption in the small intestine of rats by 35-40%. These effects were completely blocked by the antagonist hCGRP(8-37). Moreover

intravenous infusion of CGRP antagonist blocked the inhibitory effects of intraluminal capsaicin perfusion on alanine absorption (Barada *et al.* 2000). Similarly intracerebral injection of CGRP decreased alanine absorption, an effect that was reduced by vagotomy (Barada *et al.* 2000). Thus, CGRP is involved in the regulation of jejunal amino acid absorption through intrinsic (enteric) and extrinsic (central) neural mechanisms. CGRP has also been implicated in control of gastric acid secretion. Gastric acid facilitates the digestion of protein and the absorption of iron, calcium and vitamin B12. It also protects against bacterial overgrowth and enteric infections. When homeostatic mechanisms malfunction, the volume and concentration of acid may overwhelm mucosal defenses leading to duodenal ulcer, gastric ulcer and gastroesophageal reflux disease (Schubert 2003). The main peripheral stimulants of acid secretion are the hormone gastrin and the paracrine amine histamine. The main inhibitor of acid secretion is somatostasin. Somatostasin, acting via SS TR2 receptors, exert a tonic paracrine inhibitory influence on the secretion of gastrin, histamine and therefore acid secretion (Schubert 2003). CGRP stimulates somatostasin secretion and thus inhibits acid secretion. In the stomach CGRP is present in extrinsic sensory neurons. CRLR (calcitonin receptor like receptor) and RAMP 1 mRNA immunoreactivity or both are present in oxyntic and pyloric glands indicating these as the site of CGRP action. *In situ* hybridization and immunocytochemistry characterized these cells containing CRLR and RAMP 1 as somatostatin-containing D cells (Schubert 2003). In addition CGRP plays a role in gastrointestinal motility. The CGRP antagonist CGRP 8-37 has been shown to improve experimental post operative ileus in both rats (Trudel *et al.* 2002) and dogs (Trudel *et al.* 2003) as assessed by acetaminophen absorption. This suggests that CGRP plays a role in inhibition of gastric emptying and duodenal motility . This exact role of CGRP is not yet elucidated (Trudel *et al.* 2003).

Dermatological Effects

It has recently been shown that cutaneous axon terminals and epidermal melanocytes make contact via chemical synapses in human skin and that CGRP induces menalocyte proliferation (Toyoda *et al.* 1999). In vitro skin exposed to CGRP in organ

culture showed increases in melanocyte number, epidermal melanin content, melanosome number and degree of melanization (Toyoda *et al.* 1999). CGRP alone had no significant effect on melanogenesis of cultured melanocytes, whereas the addition of medium conditioned by CGRP-stimulated keratinocytes (CGRP-KCM) induced melanogenesis as indicated by biochemical assays of tyrosinase activity and melanin content (Toyoda *et al.* 1999). Furthermore, CGRP-KCM significantly enhanced melanocyte dendricity, a crucial factor affecting epidermal pigmentation. These findings suggest that keratinocytes produce and secrete some melanotropic factors following stimulation with CGRP, which modify growth, melanin synthesis and dendritic branching of melanocytes, and demonstrates intimate interactions between the cutaneous nervous system and melanocytes within the epidermal environment (Toyoda *et al.* 1999).

Pregnancy

CGRP-containing nerves extend into the reproductive organs (Yallampalli *et al.* 2002). CGRP immunoreactivity is localized to a subpopulation of myometrial afferent nerves throughout the uterine horns and cervix in rats (Yallampalli *et al.* 2002). CGRP immunoreactive (i-CGRP) nerve fibers are distributed between both the vascular and nonvascular myometrial smooth muscle components of the uterus in rats. The dense network of nerve fibers is associated with the myometrium, and the fact that one nerve fiber may innervate both longitudinal and circular smooth muscle fibers suggests an important function for this innervation in coordinating the contractility of the two muscle layers (Yallampalli *et al.* 2002). During pregnancy circulating CGRP levels become significantly increased in humans (Stevenson *et al.* 1986) and rats (Gangula *et al.* 2000b). The increase in CGRP levels begins in the third month of gestation in women, continuing until parturition, with levels increasing to 300% in the ninth month. Serum CGRP levels return to normal on the fifth day after delivery. Plasma i-CGRP levels in rats are elevated during pregnancy and decline precipitously on the day of labor at term, confirming gestational regulation of circulatory i-CGRP (Yallampalli *et al.* 2002). Because sex steroid hormones are raised during pregnancy, the increases in i-CGRP levels during pregnancy might be related to steroid hormone levels. Plasma CGRP levels are higher in

women than in men, and they are highest of all in women taking contraceptive pills (Yallampalli *et al.* 2002). Consistent with this observation injection of estradiol (E2) or progesterone (P4) into ovariectomized rats increases plasma i-CGRP concentrations (Yallampalli *et al.* 2002) In normotensive pregnant rats, the vasodilatory response to exogenous CGRP is elevated, indicating increased vasodilatory sensitivity to CGRP during pregnancy (Gangula *et al.* 1999). Studies on systemic and regional homodynamic responses to CGRP provide evidence that the increased vasodilatation after CGRP administration during pregnancy could result from decreased total vascular resistance, particularly to coronary, mesenteric and renal vascular beds, and that these effects might be sex steroid hormone dependent. Both E2 and P4 treatment of ovariectomized rats caused increased vasodilatory responses to CGRP (Gangula *et al.* 1999). In addition the increased vasodilatation appeared to result from increased CGRP-induced vascular relaxation of resistance vessels, such as the mesenteric artery. CGRP induced vascular relaxation was highest in female and lowest in males when sex steroid hormone levels were raised, indicating beneficial effects of steroid hormones (P. Laguna and C. Yallampalli, unpublished data). CGRP receptors in the mesenteric artery, identified by western immunoblotting for a monoclonal antibody against CGRP-B receptor isolated from porcine cerebellum, are raised during pregnancy (Gangula *et al.* 2000a). The CGRP-B receptor level falls at term, but are again elevated post partum when CGRP induced vascular relaxation declines, indicating perhaps that there could be multiple CGRP receptor complexes and/or changes in post receptor signaling.

Normal pregnancy is associated with an increase in uteroplacental blood flow and a decrease in uterine vascular resistance (Yallampalli *et al.* 2002). During normal pregnancy, the uterine artery is highly refractory to the potent vasoconstrictor angiotensin II, compared with that seen in non-pregnant states. Such uterine artery refractoriness results mainly from increased production of basal nitric oxide (NO) and prostaglandins in the uterine vasculature of rats, guinea pigs and sheep (Sladek *et al.* 1997). It has been shown that sex steroid hormones increase both the synthesis of CGRP and the responsiveness to synthetic CGRP in the uterine vasculature. Potent vasodilatory effects

of CGRP were also reported in the uterine arteries of sheep and humans (Yallampalli *et al.* 2002). The mechanism of action of CGRP in uterine artery relaxation is still unclear.

The placenta is another crucial organ of pregnancy, necessary for normal fetal growth and development, and responsible for the transfer of nutrients, ions and lipids from the mother into the fetus. Regulation of placental blood flow in both maternal and fetal compartments affects the transport of oxygen and nutrients, in addition to hormonal functions. Unlike other vascular systems, the fetoplacental unit lacks innervation and therefore depends on humoral substances for control of vascular tone (Yallampalli *et al.* 2002). During normal pregnancy, the net result is a fetoplacental circulation of low resistance, suggesting that vasodilator influences predominate. Besides NO and prostanoids, there are other substances yet to be identified that might induce vasodilatation and thereby play a role in the control of vascular tone in the fetoplacental unit. It has been shown that CGRP relaxes human chorionic plate vasculature (Yallampalli *et al.* 2002) and CGRP is 100 times more potent than parathyroid hormone in producing vasodilatory effects in placental cotyledons from term pregnant women (Mandsager *et al.* 1994). Pregnancy is associated with significant changes in structure and function of the uterus. The quiescent state of the uterus is essential for completion of the pregnancy. Little emphasis has been placed on the factors that might control uterine quiescence until parturition. Several studies have shown that CGRP is a potent smooth muscle relaxing factor, that may play a role in maintaining uterine quiescence (Yallampalli *et al.* 1999). CGRP has been reported to inhibit smooth muscle contractility of the uterus and fallopian tubes of women (Yallampalli *et al.* 2002). In addition, CGRP-containing nerves are present in the uterus(Shew *et al.* 1992). CGRP inhibits uterine contractility during pregnancy in the rat and this effect is dramatically decreased at term during labor. The changes in sensitivity to CGRP of the rat uterus during pregnancy and labor were associated with similar changes in CGRP-binding capacity (Yallampalli *et al.* 1999). CGRP also significantly inhibits both spontaneous and oxytocin induced contraction of the myometrium during pregnancy. The relaxing effects of CGRP were decreased in myometrium obtained from women during labor and in the non-pregnant state, indicating differential sensitivity of uterine myometrium to CGRP. To further

classify the mechanisms involved in differential sensitivity to CGRP, changing in CGRP binding and CGRP-B receptor proteins in the uterus were assessed. Binding of ^{125}I-CGRP to rat uterine membranes was increased during pregnancy and decreased at term (Yallampalli *et al.* 1999). The changes in ^{125}I-CGRP binding were associated with changes in immunoreactive CGRP-B receptors in the rat uterus, and these receptors were localized to the myometrial cells (Yallampalli *et al.* 1999). Similar to the rat, in the human myometrium CGRP-B receptors are localized to the myometrium and the changes CGRP-induced relaxation associated with gestation were also associated with changes in immunoreactive CGRP-B receptors (Dong *et al.* 1999). This up regulation of CGRP-B receptors may be regulated by sex steroid hormones. Injections of estrogen antagonist RU 468 to rats on day 18 of pregnancy substantially reduced 125i-CGRP and CGRP-B receptor protein levels in the uterus. Administration of progesterone from days 20-22 of gestation in the rat, reversed this decline in uterine CGRP-B receptors and binding (Yallampalli *et al.* 1999). Because CGRP is a potent smooth muscle relaxant, an increase in the circulatory concentration of this peptide during pregnancy and a decrease at term suggests a role for circulating CGRP in modulating uterine activity during pregnancy and labor.

Fetal Development

CGRP is also suggested to have a role in sexual maturation of the fetus. Cryptorchid animal models show abnormalities of in gubernacular contractile response to CGRP, implying that CGRP may have a key role in mediating normal inguinoscrotal testicular descent (Goh *et al.* 1993). Infusion of pregnant rats with the CGRP receptor antagonist CGRP(8-37), increased fetal mortality and decreased fetal growth (Gangula *et al.* 2002).

Pathophysiological Role of CGRP

In addition to its physiological role CGRP has been implicated in mediating responses in a number of pathological conditions. The expression of CGRP has been

assessed in the guinea pig model of allergic asthma. Changes in CGRP biosynthesis in the sensory airway innervation of ovalbumin-sensitized and –challenged guinea pigs were evaluated. A three- to four fold increase in CGRP expression along with SP was found 24 hours following allergen challenge in airway tissue (Fischer *et al.* 1996). A further study with toludene diisocyanate (TDI), a potent sensitizer that causes occupational asthma in a significant portion of subjects exposed, showed that immunization and challenge with TDI led to a decrease in CGRP- and tachtkinin-immunoreactivity in guinea pig central airways (Springer *et al.* 2003). The role that CGRP may play in asthma is mainly liked to the marked and prolonged vasodilatation which contributes to the to the hyperemia and edema found in asthmatic airways, and considered as major contributors to the narrowing of the airway lumen. CGRP also stimulates migration of eosinophils and T cells and could therefore contribute as a mediator of cellular infiltration.

Given its potent vasodilatory action, the role of CGRP in hypertensive diseases has been discussed. There is evidence that plasma concentrations of CGRP in human patients with uncomplicated essential hypertension is decreased, which contributes to the development of hypertension (Deng and Li 2005). Others however, have reported that the circulating CGRP in essential hypertension is increased (Masuda *et al.* 1992)or unchanged (Schifter *et al.* 1991). Conflicting observations might be explained by variation in the sampling methods and radioimmunoassay used, or might reflect differences caused by the heterogeneity, severity or duration of the hypertensive state, the degree of end organ damage or the treatment regime (Deng and Li 2005). In contrast to hypertension in humans, the direct role played by CGRP in some experimental models of hypertension has now been established (Deng and Li 2005). The age related decrease in neuronal CGRP expression in spontaneously hypertensive rats (SHR) and the decrease in DGR content of CGRP in Dahl-salt hypertensive rats could contribute to the development and maintenance of hypertension(Supowit *et al.* 1993). In the αCGRP knockout mice, the baseline blood pressure (BP) is significantly increased compared with the wild type mice, indicating that αCGRP plays a role in the regulation of resting BP under normal physiological conditions (Gangula *et al.* 2000c). Administration of a bolus of human αCGRP (1.5ng/g) results in a rapid 16.3 +/- 4.3 (in male) or 19.8 +/- 2.3mmHg (in

female) decrease in mean arterial blood pressure in the wild type mice and a 27.5 +/- 4.3 (in male) or 25.9 +/- 3.8mmHg (in female) reduction in the αCGRP knockout mice (Gangula *et al.* 2000c). These results suggest that a relative lack of CGRP in the knockout mice increases the responsiveness of the vasculature through a CGRP receptor-mediated mechanism (Gangula *et al.* 2000c). The following evidence also supports the notion that a decrease in neuronal CGRP contributes to the elevated BP: (1) neonatal degeneration of capsaicin-sensitive sensory neurons results in rats with an elevated BP when challenged with salt loading (Wang *et al.* 1998); and (2) intrathecal administration of capsaicin to deplete the spinal CGRP enhances the development of hypertension in rats (Burg *et al.* 1994). On the other hand, the acute administration of CGRP receptor antagonist CGRP (8-37) increases the already elevated BP in the sub-total nephrectomy-salt (SN-salt) hypertension rats(Supowit *et al.* 1997), indicating that endogenous CGRP plays a compensatory depressor role in the development of this model (Supowit *et al.* 1997).

CGRP also has an effect on vascular smooth muscle cells (Deng and Li 2005). CGRP inhibits proliferation of cultured aortic and pulmonary artery smooth muscle cells in which p53 plays a role in CGRP-induced inhibition of cell proliferation and cyclicAMP (cAMP). Phosphokinase A (PKA) appears to mediate this effect in aortic and pulmonary artery smooth muscle cells, whereas cyclicGMP (cGMP) appears to be involved in pulmonary artery smooth muscle cell proliferation (Chattergoon *et al.* 2005). Others reported that CGRP could inhibit proliferation of vascular smooth muscle cells induced by endothelin, interleukin 1 (IL-1), tumor necrosis factor-alpha (TNF-α) or angiotensin II(Li *et al.* 1997), (Qin *et al.* 2004). The mechanism by which CGRP inhibits the proliferation of vascular smooth muscle cells is related to elevations of cAMP mediated by NO, which thereby inhibits the synthesis of deoxyribonucleic acid (DNA) (Li *et al.* 1997). While these studies of vascular smooth muscle cells are conducted in vitro, it seems plausible to speculate that CGRP may inhibit the proliferation of vascular smooth muscle cells of resistance vessels in vivo (Deng and Li 2005). Since the rennin-angiotensin-aldosterone system (RAAS) is a major pro-hypertensive system, factors interacting with RAAS may be involved in the development of hypertension. A sub-

depressor dose of CGRP causes a sustained depressor in hypertensive rats induced by angiotensin II or norepinepherine (NE). CGRP also inhibits the secretion of aldosterone, especially induced by angiotensin II (Murakami *et al.* 1991). In addition, a new rat model of hypertension has been developed, in which hypertension was induced by a salt loading in rats treated with capsaicin on the first and second days of life to degenerate the capsaicin-sensitive sensory (CGRP-containing) nerves. The mechanisms responsible for the elevated BP are related to insufficient suppression of plasma renin activity and plasma aldosterone levels when challenged with salt loading, indicating that intact CGRP containing nerves are necessary for the normal natriuretic response to salt loading (Wang *et al.* 1998). Also, an increase in the activity of the circulating RAAS is observed in αCGRP knockout mice, which may contribute to the increase in BP in this model (Deng and Li 2005). On the other hand , angiotensin II pre-junctionally inhibits neurotransmission of CGRP-containing nerves through angiotensin II receptors in the mesenteric arteries of spontaneously hypertensive rats (SHR). Chronic treatment of SHR with captopril or temocapril (angiotensin converting enzyme inhibitors) restores the vasodilator response mediated by CGRP-containing nerves in the mesenteric arteries(Kawasaki *et al.* 1999). This treatment also increases the density of CGRP-containing nerves in mesenteric arteries and the level of CGRP mRNA in the DRG (Kawasaki *et al.* 1999). The aforementioned studies indicate that endogenous CGRP plays a role in the modulation of BP via interactions with RAAS. In rat mesenteric resistance arteries, inhibition of CGRP release from capsaicin sensitive nerves or CGRP (8-37) also potentiates adrenergic nerve mediated vasoconstriction without changing neuronal release of NE, suggesting that nerves that release CGRP post-synaptically inhibit sympathetic nerve mediated vasoconstriction(Takenaga and Kawasaki 1999). αCGRP contributes to the regulation of cardiovascular function through inhibitory modulation of sympathetic nervous activity (Kurihara *et al.* 2003), but NE exerts an inhibitory influence on CGRP release from CGRPergic nerves via activating pre-synaptic α2-adrenoreceptors in rat mesenteric arteries (Kawasaki *et al.* 1990). In addition, neuropeptide Y, a vasoconstrictor neuropeptide co localized with NE in peripheral adrenergic nerves, inhibits the neurogenic vasodilatation and CGRP release in mesenteric

arteries (Kawasaki *et al.* 1991). Taken together, these studies suggest that CGRPergic and adrenergic nerves counteract each other to regulate the vascular tone.

Migraine is a common chronic, paroxysmal neurobiological disorder that ranks amongst the world's most disabling medical disorders (Arulmani *et al.* 2004). The characteristic features of migraine include (i) recurrent attacks of unilateral headache, (ii) anorexia, nausea and vomiting and (iii) autonomic dysfunction (Silberstein 2004). The current theories of the pathophysiolgical bases of migraine suggest that migraine may involve dilation of the cranial blood vessels including carotid arteriovenous anastamoses and activation of the trigeminovascular system (Arulmani *et al.* 2004). Stimulation of the trigeminovascular system (trigeminal ganglia and sensory nerves) in several species leads to release of CGRP, which dilates cranial blood vessels and stimulates sensory nerve transmission (Edvinsson 2004). Moreover CGRP-like immunoreactivity is abundantly expressed in trigeminal nuclei as well as in non-myelinated trigeminal sensory nerve fibers. Thus, it is clear that the cerebral vasculature is preferentially innervated by CGRP-containing sensory nerves (Edvinsson 2004). Neurotransmitter release in cranial outflow has been explored in migraine (Edvinsson 2004), (Goadsby *et al.* 2002). Plasma concentrations of CGRP in the venous blood, but not of other neuropeptides , were elevated in the headache phase of migraine (Goadsby *et al.* 2002). Furthermore, in migraine patients: (i) a strong correlation was found between plasma CGRP concentrations and migraine headache (Juhasz *et al.* 2003), (ii) intravenous infusion of CGRP produced a migraine like headache (Lassen *et al.* 2002), (iii) baseline CGRP levels are considerably higher (Juhasz *et al.* 2003), and (iv) the changes in plasma CGRP levels during migraine attacks significantly correlated with the headache intensity (Juhasz *et al.* 2003). Recent investigations have shown that NO, a potent vasodilator implicated in migraine headache has a strong correlation with CGRP (Juhasz *et al.* 2003). Migraineurs are supersensitive to NO and the vascular effects of NO are partly mediated by CGRP release from trigeminal nerve fibers, while at the level of the trigeminal system, nitric oxide synthase coordinates with NO production to release CGRP from trigeminal nerve fibers (Akerman *et al.* 2002). Intravenous infusion of NO produces a migraine-like headache with an associated increase in plasma CRGP levels (Juhasz *et al.* 2003). This

supports the contention that the trigeminal CGRP release is a reliable marker for migraine that can be measured in a venous blood sample (Juhasz *et al.* 2003); accordingly, the decrease in this marker seems to be highly predictive of antimigraine activity in humans (Edvinsson 2004).

Chapter 2 Mechanisms of Sepsis

Introduction

Sepsis is due to a systemic inflammatory response to both infectious and non-infectious disorders; when this causes hypotension and organ dysfunction, septic shock occurs. Mortality in sepsis is due to multiple organ dysfunction. The early stages of sepsis are characterized by excessive production of inflammatory mediators (McKenzie and Furr 2001). These inflammatory mediators are numerous in number and products of activated inflammatory cells, endothelial cells and from activation of the complement and coagulation cascades. Many of these molecules have been evaluated as predictors of severity and prognosticators of survival. (Das 2006)

Definitions of Sepsis

A consensus panel in 1992 defined SIRS in humans as widespread inflammation (or clinical response to that inflammation), that can occur in patients with such diverse disorders as infection, pancreatitis, ischemia, multiple trauma, hemorrhagic shock, and immunologically mediated organ injury (Bone *et al.* 1992). The term sepsis, a subcategory of the dysfunction defined as SIRS, should be used for those patients with documented infection. The systemic inflammatory response is manifested by two or more of the following conditions: temperature>38^{o}C or <36^{o}C; heart rate >90 beats per minute (bpm); respiratory rate >20 breaths per minute or $PaCO_2$ <32 torr (<4.3kPa); white blood cell (WBC) count> 12,000cells/mm^3, <4,000 cells/mm^3 or > 10% immature (band) forms (Bone *et al.* 1992). In addition the consensus conference also recognized a progression in the disease state from simple SIRS/sepsis to severe SIRS/sepsis in the presence of acute organ dysfunction, hypotension or perfusion. This severe sepsis syndrome was defined as SIRS with one of the following manifestations of inadequate organ function/perfusion: alteration in mental status, hypoxemia (PaO2) <72 torr breathing room air; or oliguria (urine output <30mls/or 0.5ml/kg for at least 1 hour) (Bone *et al.* 1992) The next step in

progression is 'SIRS shock' present in patients with SIRS and hypotension that is unresponsive to resuscitation with fluids (Bone *et al.* 1992).

Multiple organ dysfunction syndrome (MODS) is the recognized diminished organ function associated with acute illness, in which organ function is not able to maintain homeostasis. The dysfunction may be absolute or relative but is more readily identifiable as a gradual change over time (Nystrom 1998). An example of relative organ dysfunction is the patient who has a normal cardiac output and systemic oxygen delivery but inadequate tissue oxygenation (e.g. lactic acidosis). It is further recognized that organ dysfunction may be as a direct result of a well-defined insult. The secondary form of organ dysfunction is an integral part of SIRS itself.

The clinical signs of sepsis, such as fever, tachycardia , tachypnea and leucocytosis, are common responses to systemic infection. A trigger-response concept of sepsis has emerged, in which bacteria are as seen as the trigger, with the pathophysiology being the response to that trigger. It is now recognized that sepsis involves both microbial and pathophysiological events. Uninfected trauma patients and those with intra-abdominal infection have similar clinical courses, both groups developing multiple organ failure with identical microscopic pathology- autodestructive inflammation, that seems independent of infection (Goris *et al.* 1985). Others found that the clinical response persisted after eradication of the infection and was itself associated with increased mortality (Marshall and Sweeney 1990). In animal studies it was found that the severity of the physiological response was a better predictor of outcome than the microbial challenge (Jonsson *et al.* 1993; Nieuwenhuijzen *et al.* 1993).

Sepsis and Mortality

SIRS, sepsis and septic shock are major causes of morbidity and mortality in human intensive care units (ICU) (Brun-Buisson *et al.* 2004). The incidence of bacteraemia in human hospitals has been increasing steadily over the years. In 1990, the US national center for health statistics reported that the rate of bacteraemia had increased

from 0.74/1,000 to 1.76/1,000 hospital discharges between 1979 and 1987 (1990). Much if not all of this change was caused by the increasing importance of nosocomial infection. At one tertiary care institution, the rate of nosocomial bacteraemia increased steadily from 6.7/1,000 to 18.4/1,000 discharges between 1980 and 1992 (Brun-Buisson 2000). In a French multi center study in 1993 an overall incidence rate of bacteraemia of 9.8/ 1,000 admissions was recorded. This rate was eight fold higher in the ICUs (69/1,000) than in wards (8.2/1,000) (Brun-Buisson *et al.* 1996). Extrapolating these results to the whole country would give a figure of approximately 67,500 bacteremic episodes occurring annually. So far, the most comprehensive study of the clinical significance of the early stages of the sepsis syndrome in humans was conducted by Rangel-Fausto et al, at the University of Iowa Hospital and Clinics (Rangel-Frausto *et al.* 1995). This study was performed in three ICUs (medical, surgical and cardiovascular) and three wards of a 900-bed teaching hospital. The incidence of SIRS, sepsis and severe sepsis and septic shock was assessed during a 9 month period. Of the 3708 patients admitted during the study period, 2527 (68%) met at least two criteria for SIRS at some point in their hospital stay. The major finding from this study was that medical or surgical ICU patients met 2 or more SIRS criteria during >80% of their unit stay, whereas patients in the cardiovascular ICU met such criteria over one half of their unit stay, and patients from other wards from 32% to 67% of their stay. Of the 2729 episodes of septic syndromes (i.e. at least SIRS) recorded in the incidence study, 1541 (56.4%) were classified as sepsis, 994 (36.4%) as severe sepsis and 194 (7.1%) as septic shock (Rangel-Frausto *et al.* 1995). Patients with infection were classified as having culture proven or culture-negative sepsis. Less than 50% of all episodes were microbially documented, although this proportion increased from 42% when patients only met the criteria for SIRS to 57% in patients presenting with shock. Importantly this study confirmed the expected natural progression between the different stages of septic syndromes: 32% and 36% of patients having 2 or 3 SIRS criteria, respectively developed culture proven sepsis by day 14 and 45% of those with 4 criteria subsequently developed sepsis, while 64% of those with sepsis developed severe sepsis, a median of only 1 day after sepsis; conversely, only 23% of patients presenting with severe sepsis developed septic shock, and this occurrence was delayed by a median of 28 days after severe sepsis (Rangel-Frausto *et al.* 1995). The authors also noted an

increasing prevalence of eventual organ dysfunctions (respiratory, renal, disseminated intravascular coagulation [DIC] and shock) with increasing number of SIRS criteria. The overall rates of organ failure and mortality were similar with each stage, regardless of whether infection was documented or not, with the notable exception of acute renal failure, which was more frequent at all stages , in which the presence of infection was confirmed (Rangel-Frausto *et al.* 1995). The conclusions drawn from this study were (1): The incidence of SIRS is very high in ICU patients, and its recognition cannot help in accurately identifying patients who will prove to be infected or those at higher risk of the more severe stages. This is confirmed by the fact that only about one third to one half of patients meeting SIRS criteria were subsequently proven to have confirmed (i.e. microbiologically proven) sepsis; the prevalence of infection, however, increases with the number of SIRS criteria met. However, this conclusion must be tempered by the fact that many patients with SIRS were though to have infection, and were thus administered empirically antimicrobial therapy, which likely interfered with the documentation of infection; the actual proportion of non-infectious SIRS, or 'severe SIRS' in this study is unknown. (2): There is indeed a continuum between the different stages of the inflammatory response from SIRS to sepsis, severe sepsis and septic shock. (3): Whether infection is confirmed or not, the outcomes are similar in terms of organ dysfunctions and mortality, with each corresponding stage (with the exception of renal failure). Other studies have confirmed the very high incidence of SIRS in various categories of ICU patients (Rangel-Frausto *et al.* 1995). When considering the syndromes of severe sepsis and septic shock, these syndromes are of more concern to intensivists than SIRS, given their more severe outcome, and the poor specificity (and sub-optimal sensitivity) of the latter. Two large multi-center multi-institutional hospital wide studies looked closely at these two syndromes. One, evaluating 24 hospitals on one hand (Brun-Buisson *et al.* 1996) and 170 ICUs on the other (Brun-Buisson *et al.* 1995), both surveyed during a two month period, the overall incidence of severe sepsis and shock (including clinically and microbiologically documented infection) was 6/1,000 of all hospital admissions, but only 2.9/1,000 in medical/surgical wards and 119/1,000 in ICUs. In the parallel larger ICU survey, severe sepsis or shock occurred in 9% ICU admissions; 71% of the 1064 episodes were microbiologically documented. The attack rate was higher in larger (>400 beds)

than smaller hospitals (10.3 vs. 6.7/1,000 admissions). Septic shock occurred in 6.3/1,000 ICU admissions.

Major changes have occurred in the past two decades in the epidemiology of bacteremia (Brun-Buisson 2000). These include increasing rates overall, and a growing importance of gram positive organisms over the years, especially among nosocomial episodes, which account for most of the increased rates (Pittet and Wenzel 1995). Much of this increasing role of gram-positive organisms is due to catheter-related infections and primary bacteraemia. As a result, gram-positives now outweigh gram-negatives among bacteraemic episodes (55% vs. 45%) (Brun-Buisson *et al.* 1996). In severe sepsis, however, the proportion of gram-positives and gram-negatives appear similar, reflecting the lower risk of severe sepsis associated with coagulase-negative *staphylococci*; in non-bacteraemic severe sepsis, however, gram-negative organisms appear to predominate again (Sands *et al.* 1997). There was no major difference in the distribution of organisms when comparing bacteraemic episodes associated with sepsis only or with severe sepsis. These data suggest that the microbiologic characteristics of infection are not a major determinant of the clinical presentation and intensity of the host response to infection (Brun-Buisson 2000). This is also consistent with the fact that it is quite difficult to predict bacteraemia in patients presenting with clinical sepsis (Bates *et al.* 1997).

It is apparent that the classification into three major syndromes (sepsis, severe sepsis and sepsis shock) reflects a grading in prognosis of affected patients, and this is clearly an important outcome of the current classification. There are wide variations in mortality rates reported in cohorts of human patients with septic syndromes, especially for hospital-wide data (Brun-Buisson 2000). In the study by Rangel-Fausto (Rangel-Frausto *et al.* 1995), the 28 day mortality of the different stages of SIRS to septic shock was 7%, 16% 20% and 46% for SIRS, sepsis, severe sepsis and septic shock, respectively. In the French multi-center study, the 28 day mortality was 25% in patients with bacterial sepsis and 54% in patients with severe sepsis or septic shock (Brun-Buisson *et al.* 1996).

The incidence of SIRS, sepsis, severe sepsis and septic shock in hospitalized patients has not been reported in the equine literature. However, data does exist with regard to neonatal foals (<2 weeks of age), in which sepsis is recognized as a major cause of death (Cohen 1994). The exact incidence of neonatal sepsis has not been reported (Roy 2004), due to the fact that prediction of documented septicemia is difficult. In a study conducted by Wilson and Madigan septicemia was confirmed by bacteriological culture of necropsy samples in 32 foals < 8 days old, and compared to antemortem blood samples submitted for culture (Wilson and Madigan 1989). Blood failed to yield any gram negative organisms in 12 (37.5%) of 32 foals. 10% of the gram positive bacteria found at necropsy were not detected earlier by results of bacteriologic culture of blood (Wilson and Madigan 1989). In addition blood culture results may require 7 days before results are obtained and delay in treatment is an independent risk factor for poor survival (Gayle *et al.* 1998). Attempts to identify neonates with septicemia have included development of a sepsis scoring system that combines historical and clinicopathological data, and subjective measures to derive a numerical representation of the patient's condition (Brewer and Koterba 1988). A calculated sepsis score of >12 in foals less than 12 days of age predicted sepsis with a sensitivity and specificity of 93% and 83% respectively (Brewer and Koterba 1988). When this scoring system was re-evaluated by Corley and Furr in a different hospital population of foals of the same age, positive predictive value was reported as 93% and negative predictive value of only 53% (Corley and Furr 2003). The authors concluded that this low negative predictive value limited the clinical utility of the scoring system. Although bacterial infection may be responsible for the initiation of the inflammatory response, the inflammatory process itself results solely from the production of endogenous mediators (McKenzie III and Furr 2001). This explains the limited utility of the sepsis score, since the inflammatory response is responsible for many of the changes seen in clinical and clinicopathological data used in the scoring system, regardless of the initiating cause of the inflammatory response.

Pathophysiology of Sepsis

Inflammation represents the response of tissues to either injury on the presence of microorganisms. The first step in this process is the recognition of tissue injury or microbial invasion (McKenzie III and Furr 2001). Injured cells release preformed mediators and synthesize proinflammatory substances, including eicosanoids (e.g. prostaglandins, thromboxanes and leukotrienes) and the cytokines (interleukin [IL]-1 and tumor necrosis factor (TNF)-α. These mediators are responsible for the initiation of a non-specific inflammatory response (McKenzie III and Furr 2001). The bacterial cellular components that are recognized by the immune system include endotoxins (lipopolysaccharide; LPS) and exotoxins from gram-negative bacteria as well as peptidoglycans (PGs), lipotechoic acids (LTAs), enterotoxins, and superantigenic exotoxins from gram positive bacteria (Teti 1999), (Woltmann *et al.* 1998).

On entering the circulation LPS is avidly bound by LPS-binding protein (LBP); the LPS-LBP complex then binds to a receptor present on the surface of the mononuclear phagocyte (mCD14) or in the circulation (sCD14) (Woltmann *et al.* 1998). CD14 also binds PG and LTA from gram positive bacteria and this may represent the route for cellular activation in gram-positive infections (Das 2000). The LPS-LBP CD14 or PG-LTA-CD14 complex is then responsible for activation of the mononuclear phagocyte via the toll-like receptor (TLR). Cellular activation may also occur because of the development of a non-specific oxidative stress reaction within the mononuclear phagocyte following stimulation by pro-inflammatory stimuli such as TNF-α, endotoxin or exotoxins (Woltmann *et al.* 1998). The development of the inflammatory response is dependent on the production, from the activated mononuclear phagocyte, of numerous inflammatory mediators. The transcription of many of the genes encoded for these mediators or the enzymes that produce them is dependent on the transcription activator nuclear factor-κB (Woltmann *et al.* 1998). These inflammatory mediators include proinflammatory cytokines (e.g., TNF-α, Il-1, Il-6), proinflammatory enzymes (e.g., inducible NOS, phospholipase A2, cyclooxygenase -2), and adhesion molecules (e.g., selectins, intracellular adhesion molecules) (Adrie and Pinsky 2000).

The initial changes that occur in an inflammatory response are primarily a result of local vasodilatation and increased vascular permeability. Changes occur in the vascular endothelium under the influence of molecules arising from the injured tissues, resulting in neutrophil diapedesis and increased vascular permeability (Das 2000). On arriving at the site of tissue injury, neutrophils and macrophages phagocytose foreign material and dead tissue cells and destroy it by oxidative mechanisms (neutrophils) and oxidative and non-oxidative mechanisms (macrophages). In addition macrophages release a number of factors to augment the inflammatory process including Il-1, TNF-α, Il-6, IL-12 and IL-18. These pro-inflammatory cytokines target neutrophils to increase their production of secondary inflammatory mediators including phospholipase derivatives and reactive oxygen species (Woltmann *et al.* 1998). This further increases the activity of the inflammatory response. Il-1, Il-6 and TNF-α are also important in many phases of the response to inflammatory stimuli, including complement activation, coagulation and fibrinolysis (Woltmann *et al.* 1998). Also included in the acute phase response is a counter regulatory anti-inflammatory component that normally functions to minimize and resolve the inflammatory response to localized stimuli. This counter regulatory response consists of anti-inflammatory mediators that inhibit macrophage activation (e.g, IL-4, IL-10, Il-13, adrenal corticosteroids, transforming growth factor-β, prostaglandin-E2) and soluble receptors of proinflammatory cytokines (e.g., soluble IL-1 receptor) (Pinsky 2004). The balance between pro- and anti-inflammatory components is important in determining the characteristics of the inflammatory response (Pinsky 2004). Excessive activity of the anti-inflammatory component may lead to immunosupression during or after SIRS which has been termed the compensatory anti-inflammatory response syndrome (CARS) (Bone *et al.* 1992). In moderation the changes associated with the inflammatory response are protective, resulting in enhanced killing of microbes and increased tissue healing. However, excessive malignant pro-inflammatory response leads to the previously described SIRS, severe sepsis and septic shock. One of the first effects that occurs with SIRS is widespread endothelial activation. These activated cells produce inflammatory cytokines and increased amounts of NO, prostaglandins and endothelin I(Wort and Evans 1999), and retract from each other increasing the size of the

intercellular pores and thereby increasing vascular permeability. The production of prostanoids, particularly thromboxane A2 results in pulmonary vasoconstriction leading to pulmonary hypertension (Lavoie *et al.* 1990). The initial hypertensive phase is followed by systemic hypotension caused by decreased arterial tone, decreased left ventricular preload, combined with venous dilation in the large-capacity vessels that decreases venous return. These effects are likely due to prostacyclin and NO (Wort and Evans 1999) and can progress to the syndrome of hyperdynamic shock with increases in heart rate and cardiac output developing as compensatory mechanisms to maintain tissue perfusion. This compensatory response is impaired by the reduction in left ventricular preload and decreased cardiac contractility resulting from myocardial depressants (NO, TNF-α, IL-1) (Astiz and Rackow 1998). Changes occurring in the microvasculature further contribute to the impairment in tissue perfusion. Arteriolar vasoconstriction develops due to impairment of the normal autoregulatory systems (NO from NOS, PGI_2 from cyclooxygenase), caused by inflammatory cytokines and endothelin-1, combined with an increased production of vasoconstrictive substances (endothelin-1,thromboxane A_2). Adherence of neutrophils to the endothelium further impairs blood flow. Accumulation of fibrin and aggregation of platelets and erythrocytes secondary to activation of the clotting system causes occlusion of the vasculature leading to tissue hypoperfusion. Increased vascular permeability leads to fluid extravasation and further contributes to hypotension and hypovolemia (McKenzie III and Furr 2001). Progressive alteration of the microcirculation leading to failure may represent the final common pathway of SIRS-related injury contributing to MODS (Woltmann *et al.* 1998).

CGRP and the pathophysiology of sepsis

Since abnormalities in cardiovascular tone are of paramount importance in the development of sepsis and septic shock, much work has been focused on the role of CGRP in sepsis, both as an instigator of the abnormalities in vascular tone and as possible marker in predicting sepsis or mortality associated with sepsis (Beer *et al.* 2002). CGRP levels are increased in patients with sepsis (Beer *et al.* 2002), (Joyce *et al.* 1990a), (Arden *et al.* 1994), (Arnalich *et al.* 1996), (Griffin *et al.* 1992). A study of 26 healthy

human volunteers and 16 patients with sepsis, plasma CGRP levels were elevated in the patients with sepsis, 14.9+/1 3.2pg/ml, compared to plasma CGRP levels in the healthy volunteers, 2.0 +/- 0.3pg/ml ($p<0.0005$) (Joyce *et al.* 1990a). A study by Arnalich et al. evaluating 22 patients with confirmed sepsis found that from the day of admission, septic shock patients had significantly higher CGRP-like immunoreactivity levels than patients with sepsis, as well as both groups when compared to control subjects (Arnalich *et al.* 1995). Plasma CGRP levels at study entry correlated well with the APACHE II score ($r=0.71$, $p<0.01$). Studies in dogs have shown that infusing 100, 200 and 600 pg/ml of CGRP for 10 minutes resulted in plasma CGRP levels that were increased by twofold, fivefold, and eightfold, respectively, over basal levels (Joyce *et al.* 1990b). These elevated CGRP levels were associated with decreases in mean arterial pressure from control levels of 89mmHg to 76, 67 and 55mmHg respectively (Joyce *et al.* 1990b). Patients with sepsis in the study by Joyce et al demonstrated a 7.5 fold increase in plasma CGRP levels compared to healthy volunteers (Joyce *et al.* 1990a). The elevated CGRP levels and the altered hemodynamic profiles in these patients corresponded to animal models with similar CGRP and hemodynamic profiles. These correlations provide evidence that CGRP may be a mediator of the hyperdynamic state in sepsis (Joyce *et al.* 1990a). CGRP vasodilates, decreases systemic vascular resistance (SVR), causes hypotension and may have positive chronotropic and inotropic effects (Joyce *et al.* 1990a; Tippins *et al.* 1984). These are the hemodynamic effects that are seen in sepsis. A similar effect has been seen in rats under conditions of experimental sepsis (Huttemeier *et al.* 1993). Exposure of rats to 8mg/kg bwt of endotoxin caused hypotension and tachycardia within 60 minutes of administration (Huttemeier *et al.* 1993). After two hours more severe hypotension developed and 80% rats died spontaneously after 3 hours. In other endotoxic rats the administration of 20nmols of the CGRP receptor antagonist CGRP(8-37) intravenously 60 minutes after endotoxin exposure transiently reversed the tachycardia (from 469 +/- 11 to 407 +/- 7 beats, $P<0.05$) and increased mean blood pressure (from 63+/- 4 to 93 +/- 11mmHg, $p<0.05$) over 30 minutes (Huttemeier *et al.* 1993). Survival rates were no different for treated or untreated animals. Plasma CGRP concentrations in non-endotoxemic rats were initially 30.5 +/- 3.3pg/ml and were significantly increased to 63.7 +/- 4.6pg/ml 2 hours after induction of endotoxemia with

3mg/kg *E.coli* endotoxin ($p<0.001$; n=13) (Griffin *et al.* 1992). A higher dose of LPS did not further elevate plasma CGRP levels. CGRP levels in abdominal aorta, inferior vena cava, stomach, kidney and left ventricular myocardium were not changed significantly following injection of endotoxin. However, in lung and mesenteric artery the levels increased significantly from 1.47 +/- 0.12 and 7.97 +/- 1.32pmol/g wet weight tissue to 1.96 +/- 0.19 ($p<0.05$, n=11) and 15.02 +/- 2.3pmol/g ($p<0.01$; n=7) respectively. The changes in plasma concentration and tissue content of CGRP suggest that splanchnic organs may be the source of elevated CGRP in endotoxemia (Griffin *et al.* 1992). This was confirmed by Zhou et al who evaluated CGRP levels after cecal ligation and puncture (CLP) in rats (Zhou *et al.* 2001). Plasma levels of CGRP were measured at 2, 5 and 10 hours after CLP (early hyperdynamic sepsis) and 20hours after CLP (late hypodynamic sepsis). Plasma CGRP increased by 177% 10 hours after CLP ($p<0.05$). At 20 hours after the onset of sepsis, however, the elevated plasma CGRP returned to the sham level. In samples of small intestine, liver, spleen, lung and heart sampled at 10 hours, only the small intestine showed a significant increase in tissue levels of CGRP (by 129%, $P< 0.05$) (Zhou *et al.* 2001). Immunohistochemistry examination revealed that CGRP-positive staining increased in the intestinal tissue at 10h after onset of sepsis. The distribution of CGRP staining was associated with intestinal nerve fibers. These results show that upregulation of CGRP occurs transiently during the progression of sepsis (at the late phase of hyperdynamic sepsis), and the gut appears to be a major source of such an increase in circulating levels of this peptide (Zhou *et al.* 2001).

In addition to its vascular effects CGRP exerts an effect on inflammatory cells (Springer *et al.* 2003). Inhalation of CGRP induced eosinophilic inflammation in rat lung (Bellibas 1996). CGRP has also been demonstrated to stimulate the beta integrin-mediated T-cell adhesion to fibronectin, a major glycoprotein component of the extracellular matrix, which is important for T-cell migration to inflamed sites (Levite *et al.* 1998). CGRP has also been shown to inhibit macrophage secretion and macrophage capacity to activate T cells (Nong *et al.* 1989). These effects indicate a potent anti-inflammatory property of the peptide (Springer *et al.* 2003). CGRP has also been shown to directly suppress Il-2 production and proliferation in murine T cells (Wang *et al.*

1992). CGRP-immunoreactive nerve fibers were found in close contact to dendritic cells and CGRP exerted suppressive effects on the activation of dendritic cells (Carucci *et al.* 2000). The mode of signaling by which CGRP exerts its immunomodulatory effects has not been investigated do date (Springer *et al.* 2003). CGRP also causes a dose dependent decrease of LPS induced rise in CD11b expression of activated monocytes and neutrophils, one of the major intergrins involved in neutrophil and monocyte chemotaxis (Monneret *et al.* 2003). Stimulation of dendritic cells with CGRP reduces their expression of major histocompatability class II (MHC II) and co-stimulatory B7 proteins, augments the production on IL-10 and thereby inhibits their antigen presenting capacity (Carucci *et al.* 2000; Fox *et al.* 1997; Hosoi *et al.* 1993; Liu *et al.* 2000; Monneret *et al.* 2000). In contrast to the reported anti-inflammatory properties of CGRP, a recent study has demonstrated the potential of CGRP to stimulate the release of inflammatory cytokines in a human bronchial epithelial cell line. Exposure of BEAS-2B cell line to CGRP in a range of concentrations resulted in immediate increases in intracellular calcium, the synthesis of transcripts for the inflammatory cytokines, IL-6, IL- 8 and TNF-α after 2h of exposure and the release of their protein after 6h of exposure (Veronesi *et al.* 1999). Given the myriad of pro and anti- inflammatory molecules involved in the pathogenesis of SIRS, it is hard to assign individual pathological variables to individual markers, thereby explaining the difficulty of selecting individual targets for therapy or as prognostic indicators.

Markers of Disease Severity

Many proinflammatory molecules have been evaluated as possible markers of mortality or morbidity. These include procalcitonin (Guven *et al.* 2002), serum amyloid A (Huttunen *et al.* 2003) and interleukin-1 (Marie *et al.* 2000). Procalcitonin is recognized as a useful marker in predicting severity of illness and mortality in critically ill patients (Giamarellos-Bourboulis *et al.* 2002; Hatherill *et al.* 2000; Meisner *et al.* 1999; Reinhart *et al.* 2000). Evaluation of current meta-analyses regarding predictors of mortality by Schetz found that the area under the receiver operating curve for procalcitonin was higher than for the APACHE III scoring system, the most commonly

used scoring system in human medicine for predicting mortality associated with critical illness (Schetz and Van den Berghe 2005) . The related molecule of the CALC-1 gene CGRP has received less interest in the human literature. However in addition to its pro- and anti-inflammatory role, plasma concentration of CGRP can act as predictive indicators for lethal outcome in cases of sepsis (Beer *et al.* 2002). In a study of 61 patients with confirmed sepsis after major visceral surgery, CGRP levels were significantly higher in non-survivors than in survivors as early as day one of sepsis and remained significantly elevated in non-survivors throughout the entire course of sepsis ($p<0.05$) (Beer *et al.* 2002). On the basis of the immunological activities of CGRP, the early increase of CGRP in sepsis, and the correlation of high systemic levels with outcome, it is speculated that the neuroendocrine system may contribute to monocyte deactivation in sepsis through the release of CGRP (Beer *et al.* 2002). The measurement of both CGRP and procalcitonin in equidae with critical illness is therefore of interest as both a marker of severity and as a predictor of mortality.

Chapter 3: Measurement of CGRP

Introduction

Both procalcitonin and CGRP have been evaluated as markers of severity and mortality in human patients. No literature exists on either molecule in the equid. The gene sequence of procalcitonin and CGRP were established in equidae by Toribio in 2003 (Toribio *et al.* 2003). Amino acid homology between equine and human procalcitonin is 74% and 68% between equine and rat procalcitonin. The homology between equine CGRP II and human CGRP I is 86% and 94% between equine CGRP II and rat CGRP I. This suggested that rat CGRP-I represented the best target molecule for investigation of equine CGRP.

Detection of CRGP

Serum or plasma CGRP concentrations may be measured via radioimmunoassay (RIA) (Arden *et al.* 1994; Griffin *et al.* 1992; Joyce *et al.* 1990a; Zhou *et al.* 2001), or by enzyme linked immunoassay (ELISA) (Frobert *et al.* 1999). To measure plasma CGRP concentrations using the RIA, ^{125}I-CGRP is used along with anti-rat antibody, to determine the CGRP concentration using a gamma counter (Zhou *et al.* 2001). The manufactured ELISA kit uses a double sandwich antibody technique using monoclonal antibodies to CGRP (Frobert *et al.* 1999). Research ELISA kits for rat CGRP-I were used in this study

Principles of the Enzyme Linked Immunoassay

The Enzyme Linked Immunoassay (ELISA) is based on a double-antibody sandwich technique. The wells of the microtiter plate supplied with the kit are coated with a monoclonal antibody specific for CGRP. This antibody will bind any CGRP introduced into the wells. An anticholinesterase (AChE) – Fab' conjugate (anticholinesterase labeled with a fluorescent antibody) which binds selectively to a

different epitope on the CGRP molecule is also added to the wells. This allows the two antibodies to form a sandwich by binding to different parts of the CGRP molecule. The sandwich is immobilized on the plate so that excess reagents may be washed away. The concentration of the rat CGRP is then determined by measuring the enzymatic activity of the AChE using Ellman's Reagent. The AChE tracer acts on the Ellmans's Reagent to form a yellow compound. The intensity of the color, which is determined by spectrophotometry at 405nm, is proportional to the amount of rat CGRP present in the well during the immunological incubation.

Assay Characteristics

The chosen assay is an immunometric assay for rat α-CGRP[a], produced for determination of rat CGRP concentrations in a research setting. Plasma and serum concentrations of CGRP may be measured directly. CSF concentrations of CGRP may be measured after serial dilution, and tissue concentrations of CGRP may be measured after extraction. The Enzyme Immunometric assay of rat CGRP has been validated for its use in buffer and in plasma. The reported limit of detection, calculated as the concentration of CGRP corresponding to the NSB average (n=8) plus three standard deviations, was 0.7 (in EIA buffer) and 2.0 pg/ml in plasma). Reported intra- and inter- assay CVs were <10% from 50-400 pg/ml and <20% at 10 pg/ml.

Cross Reactivity

Rat CGRP α & β	100%
Human CGRP α & β	83%
CGRP 98-37	<0.01%
Substance P	<0.01%

Human CGRP-I

ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF

Rat CGRP-I

SCNTATCVTHRLAGLLSRSGGVVKDNFVPTNVGSEAF

Equine CGRP-II

SCNTATCVTHRLAGLLSRSGGVVKSNFVPTDVGSEAF

N-terminus (a.a 1-7) is the binding site for the tracer monoclonal Ab

C-terminus (a.a 24-37) in the binding site for the solid phase monoclonal Ab

Percent homology	
Rat CGRP-I to equine CGRP-II	95%
Rat CGRP-I to equine CGRP-II N-terminus	100%
Rat CGRP-I to equine CGRP-II C-terminus	85%
Human CGRP-I to equine CGRP-II	84%
Human CGRP-I to equine CGRP-II C-terminus	71%
Human CGRP-I to equine CGRP-II N-terminus	77%

Based on the greater homology at the C and N terminus monoclonal Ab binding sites between rat CGRP-I and equine CGRP-II, the rat CGRP-I test kit was though likely to be superior to the human assay for measurement of equine CGRP. This was supported by preliminary work using both rat and human ELISA kits. CGRP concentrations from serum obtained from 10 horses were consistently higher when measured with the rat ELISA kit.

Chapter 4: CGRP in Normal and Critically Ill Horses

Objectives of the Study

A) Assay Validation

1) To determine if equine CGRP was present in the serum of normal horses as detected by a commercial ELISA kit for rat αCGRP.
2) To validate this assay for measurement of equine CGRP by establishment of percentage recovery, intra- and inter-assay coefficients of variance and presence of dilutional parallelism.

B) CRGP concentration in healthy horses

3) To determine the normal concentration of CGRP in serum from healthy adult geldings and mares.
4) To determine the normal concentration of CGRP in serum from healthy foals < 2 weeks of age
5) To determine the serum concentration of CGRP in anesthetized horses exposed to a known quantity of endotoxin.

C) CGRP concentration in horses with SIRS

6) To assess whether serum CGRP concentration was altered in adult horses with signs of systemic inflammatory response syndrome.
7) To assess whether serum CGRP concentration was altered in neonatal foals less than two weeks of age with signs of systemic inflammatory response syndrome.

Material and Methods

Assay Protocol

To prepare the ELISA plate it was rinsed 5 times with 300µl/well of wash buffer. The buffer was then removed by inverting the plate and shaking out the remaining drops. The first plate column was left empty for blanking Ellman's Reagent and all samples and reagents were allowed to reach room temperature before performing the assay. Eight serial dilutions of rat CGRP were made from 500pg/ml to 3.91pg/ml, and were added in duplicate to rows 2-3 of the ELISA plate using a manual micropipetter. Clean pipette tips were used for each serial dilution. 100µl of each sample was added in triplicate to appropriate wells of the remaining plate in a linear configuration. Pipette tips were changed between samples. 100µl of anti-CGRP tracer was then dispensed into each well. The plate was covered with plastic film and incubated in the dark at 4^{o}C for 16-20 hours.

To develop the plate, it was emptied by turning over and shaking. Each well was manually washed 3 times with wash buffer, followed by slight shaking for two minutes and a further rewash 3 times with wash buffer (300µl/well). 200µl of Ellman's Reagent was then dispensed into each well. Reported stability of Ellman's Reagent was 24 hours in the dark at 4^{0}C. However obvious yellow discoloration was present in the solution after four hours, and fresh solution was reconstituted for each plate when run. The plate was then incubated in the dark (covered in aluminum foil) at room temperature for 30 minutes. As no 'stop' solution was supplied with the ELISA kit, all samples were read at exactly 30 minutes. The plate was read using a BioRad model 550 microplate reader at 405nm and log-log standard curves were calculated form the average absorbance of each pair of standard wells.

These standard curves were then used for calculation of CGRP concentrations of unknown samples. (see figure 1)

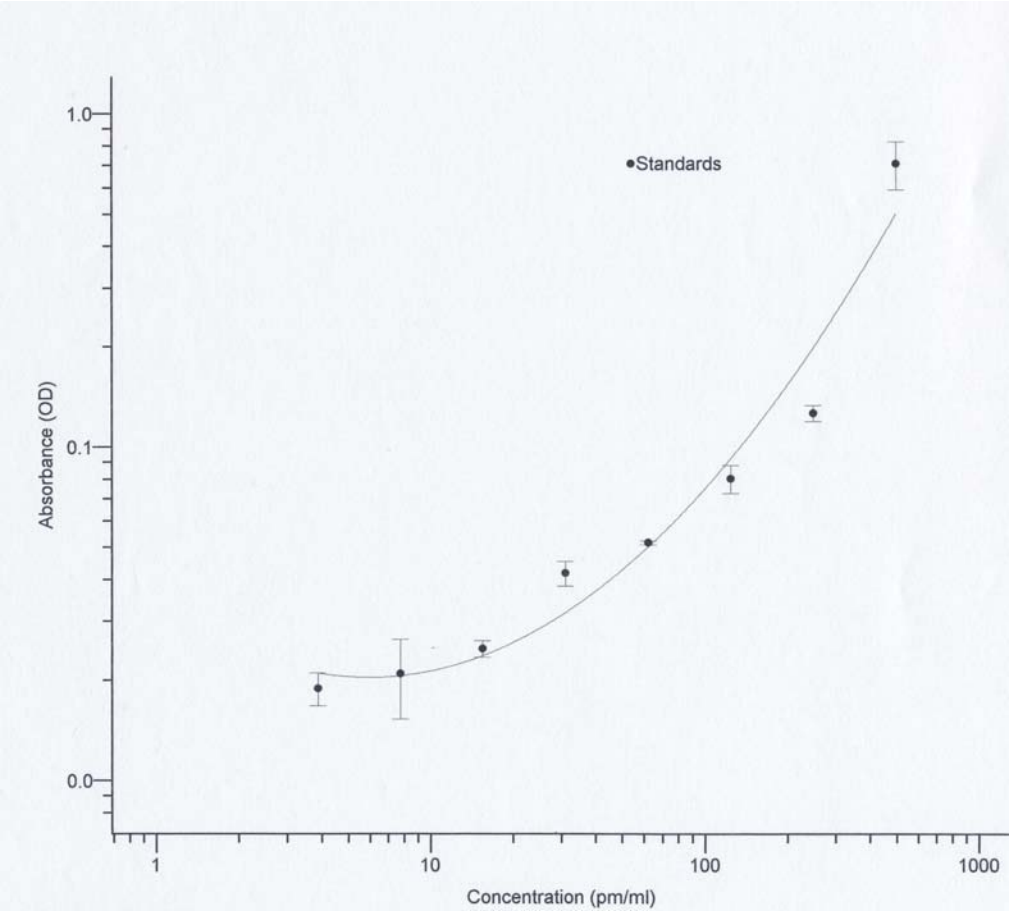

Figure 1. Example of a standard curve for measurement of CGRP concentration.

ASSAY VALIDATION

Reasons for Validation

The assay was validated to ensure that equine CGRP could be detected using the rat CGRP ELISA kit and to determine whether this measurement was repeatable both within and between assays.

Creation of CGRP free serum

CGRP free serum was created by passing equine serum through a gel column containing CGRP affinity sorbent (immobilized mouse anti-CGRP antibodies) and collecting the effluent.

Establishment of high CGRP and low CGRP pooled serum

Blood was obtained by jugular venipuncture from 30 adult horses. These horses were included horses hospitalized for a variety of medical or orthopedic problems. All samples were obtained when venipuncture was being performed for reasons related to the horse's clinical condition. Blood was collected into plain vacuum collection tubes. The serum was separated by centrifugation within 30 minutes of sample collection and one milliliter aliquots were frozen at -70^{o}C until assayed.

Duplicate samples from each horse were assessed. A standard curve of rat CGRP prepared in equine CGRP free serum was used to obtained serum CGRP concentrations for the 30 horses. Based on these results serum was pooled into 3 groups:
High CGRP (80-120pg/ml)
Medium CGRP (50-80pg/ml)
Low CGRP (<50pg/ml)

Dilutional Parallelism

Due to the inability to obtain purified equine CGRP, the parallelism of the assay was determined between rat CGRP spiked equine CGRP free serum standards, and serial dilutions of equine CGRP free serum with serial dilutions of equine serum of predetermined high, medium and low CGRP concentrations.

Intra-assay variability

Each of the above samples was run in triplicate and a coefficient of variance calculated for each data set.

Inter-assay variability

The above assay was conducted on two separate occasions 24 hours apart using different test kits and reagents.

Experimental Groups (Group data is summarized in table 1)

Group 1: Normal adult mares

Serum was obtained by jugular venipuncture from ten normal adult horses, as assessed by clinical examination and absence of abnormalities on compete blood count and biochemistry profile, and collected into plain vacuum tubes. The horses were pasture turned out with free access to water, and regularly handled daily. Samples were collected at 8am and 8pm of the same in order to determine if there was any diurnal variation in serum CGRP levels. Within 30 minutes of sample collection the serum was separated by centrifugation and frozen in 1ml aliquots at -70^{o}C. The ELISA CGRP assay previously described above was utilized to determine serum CGRP levels. Each serum sample was assayed in triplicate and the mean CGRP value was determined using a standard curve generated from equine serum standards run concurrently with the samples

Group 2: Normal adult geldings

Since CGRP concentrations in humans vary between male and females blood was obtained from a population of healthy geldings for analysis. Stored serum was obtained from 10 healthy geldings that were recorded as normal by clinical examination at the time of blood collection. This serum had been collected as part of a previous study and frozen in 1ml aliquots at -70^{o}C for <12 months. The ELISA CGRP assay previously described above was utilized to determine serum CGRP levels. Each serum sample was assayed in triplicate and the mean CGRP value was determined using a standard curve generated from equine serum standards run concurrently with the samples

Group 3: Normal equine neonates less than two weeks of age

In order to determine if CGRP concentrations are affected by age serum was obtained from a population of healthy foals for analysis. Stored serum was obtained from 10

healthy foals < two weeks of age, recorded as clinically normal at the time of blood collection. These foals were handled daily and had venipuncture performed weekly for an unrelated trial. This serum had been frozen in 1ml aliquots at -70^{o}C for <12 months. The ELISA CGRP assay previously described above was utilized to determine serum CGRP levels. Each serum sample was assayed in triplicate and the mean CGRP value was determined using a standard curve generated from equine serum standards run concurrently with the samples

Group 4: Normal adult horses exposed to endotoxin

In order to determine if serum CGRP concentrations increased acutely following a severe systemic inflammatory stimulus, a series of serum samples collected from horses exposed to endotoxin in a previous study examining the effects of hetastarch, hypertonic saline and isotonic polyionic fluids in equine endotoxic shock were assayed. The endotoxin exposure in the previous study consisted of intravenous administration of a bolus of 20µg/kg of *E.coli* O55:B5 endotoxin over one minute followed by continuous infusion of 1µg/kg/minute for 30 minutes. Serum samples were collected at 0 and 180 minutes after the end of the bolus administration and separated by centrifugation, then frozen at -70^{O}C. The ELISA CGRP assay described above was utilized to determine serum CGRP levels. Each serum sample was assayed in triplicate and the mean CGRP value was determined using a standard curve generated from equine serum standards run concurrently with the samples.

Group 5: Critically ill equine patients

Group 5a Critically ill adult horses

In order to assess the potential relationship between serum CGRP levels and clinical SIRS in equine patients, serum samples were collected prospectively from 10 adult

horses presenting with enterocolitis and signs consistent with the systemic inflammatory response syndrome. This was defined as presence of two or more of the following clinical abnormalities: fever or hypothermia (rectal temperature greater than 39.2^{o}C or less than 37.2^{o}C, leucopenia or leucocytosis (leucocyte count greater than 12,500 or less than 4,000 cells/µl), tachycardia (heart rate greater than 60 beats per minute) or tachypnea (respiratory rate greater than 36 breaths per minute) or hypocapnia (partial pressure of carbon dioxide less than 32mmHg). Samples were collected as part of the routine collection of blood samples at presentation, by venipuncture. Subsequent samples were collected 24 hours after hospitalization, in conjunction with routine clinical monitoring. All samples were separated by centrifugation and frozen at -70^{o}C until assayed as described above. Serum samples were assayed in triplicate and the mean CGRP value was determined using a standard curve generated from equine serum standards run concurrently with the samples. As sample collection coincided with collection of blood samples for routine clinical monitoring and did not involve any additional invasive procedures, client consent was not sought beyond that obtained at hospital admission.

Group 5b Critically ill foals less than two weeks of age

In order to assess the potential relationship between serum CGRP levels and clinical SIRS in equine neonates, serum samples were collected prospectively from 10 neonatal foals less than two weeks of age presenting with signs consistent with the systemic inflammatory response syndrome. This was defined as presence of two or more of the following clinical abnormalities: fever or hypothermia (rectal temperature greater than 39.2^{o}C or less than 37.2^{o}C, leucopenia or leucocytosis (leucocyte count greater than 12,500 or less than 4,000 cells/µl), tachycardia (heart rate greater than 120 beats per minute) or tachypnea (respiratory rate greater than 30 breaths per minute) or hypocapnia (partial pressure of carbon dioxide less than 32mmHg). Samples were collected as part of the routine collection of blood samples at presentation, via the indwelling intravenous catheter (as all blood samples are drawn on foals at admission by this route in our facility). Subsequent samples were collected by jugular venipuncture, 24 hours after hospitalization, in conjunction with collection of blood samples for routine clinical

monitoring. All samples were separated by centrifugation and frozen at -70^{o}C until assayed as described above. Serum samples were assayed in triplicate and the mean CGRP value was determined using a standard curve generated from equine serum standards run concurrently with the samples. As sample collection coincided with routine clinical monitoring and did not involve any additional invasive procedures, client consent was not sought beyond that obtained at hospital admission.

Statistical Analysis

Serum CGRP concentrations between groups and changes over time within groups were compared using a Paired T-Test for normally distributed continuous data and by Mann-Whitney Test for non-normally distributed data. A *p* value of less than 0.05 was considered significant. Samples values are reported as mean +/- standard error of mean (SEM)

Group	***Category***	***Number of individuals in group***	***Mean age***	***Percentage Mortality within group***
1	Healthy adult mares	18	8.9 years	N/A
2	Healthy adult geldings	11	14	N/A
3	Healthy foals	11	5 days	N/A
4	Adult horses experimentally exposed to endotoxin	12	9 years	N/A
5a	Adult horses with evidence of SIRS	11	10.1 years	73%
5b	Foals with evidence of SIRS	8	36 hours	25%

Table 1. Summary data of the five experimental groups.

Results

Assay validation

Intra-assay variability: The intrassay variability was 3.8 (+/- 0.89) %
Inter-assay variability: The interassay variability was 21.3 (+/-4.68)%
Percentage recovery of CGRP was 71.7%.

Dilutional Parallelism

CGRP free serum was spiked with equine serum containing high, medium or low quantities of CGRP and subsequently diluted in a serial fashion. Absorbance data remained parallel from 500-30pg/ml CGRP. (see figure 2)

Lower limit of detection

The lower limit of detection for this assay was determined to be 30pg/ml.

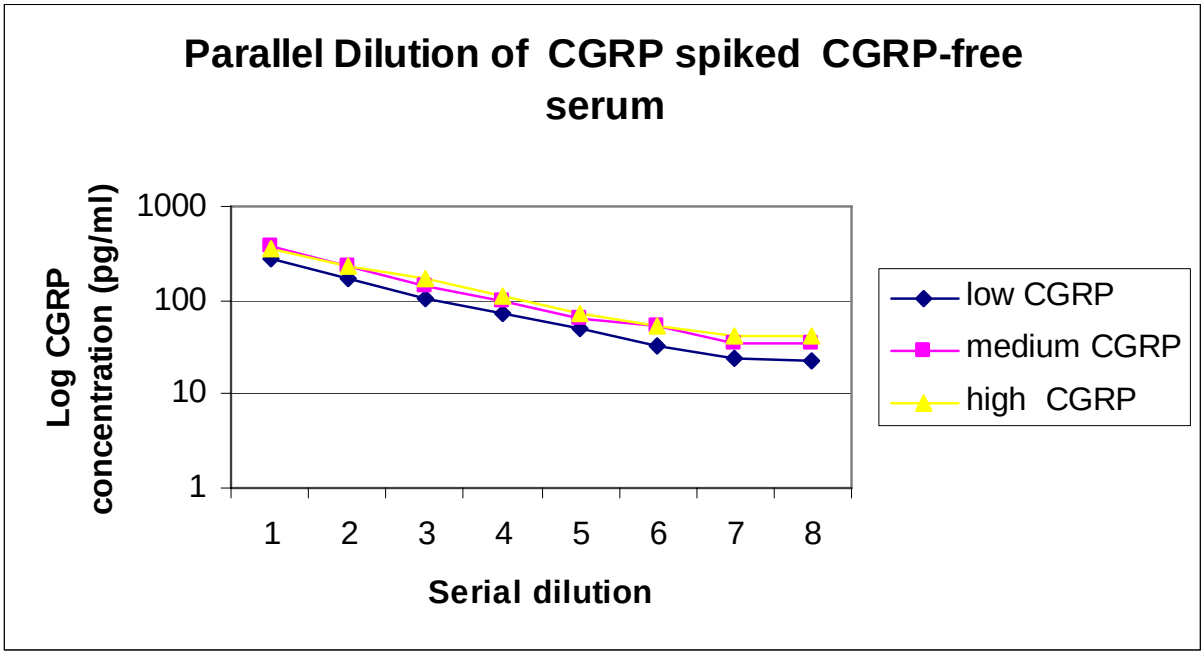

Figure 2. Log graph of parallel dilution of CGRP spiked CGRP-free serum.

CGRP concentration of healthy horses

Data was obtained from 11 healthy geldings, 18 healthy mares (9 pregnant, 9 non-pregnant) and 11 healthy foals less than two weeks of age,

In healthy adult geldings the mean serum CGRP concentration was 34.47 (+/- 5.64) pg/ml.

In healthy adult mares the mean serum CGRP concentration was 189.13 (+/- 27.85) pg/ml. Mean serum CGRP concentration was 191.00 (+/- 40.01) pg/ml at 8am and 187.28 (+/- 39.81) pg/ml at 8pm. Mean CGRP serum concentration was 209 (+/- 43.80) pg/ml in pregnant mares and 168.73(+/- 35.02) pg/ml in non-pregnant mares. Serum CGRP concentrations were higher in adult mares than adult geldings. There was no significant difference between serum CGRP concentrations in pregnant and non-pregnant mares.

In healthy foals less than two weeks of age mean serum CGRP concentration was 25.82 (+/- 6.33) pg/ml.

CGRP concentrations in horses with SIRS

Data was obtained from 12 anesthetized horses experimentally exposed to endotoxin, 11 adult horses presenting with clinical and clinicopathological evidence of SIRS and 8 foals with evidence of SIRS.

In adult horses with evidence of SIRS mean serum CGRP concentration was 116.92 (+/- 26.76) pg/ml. Mean serum CGRP concentration was 116.05 (+/- 38.58) pg/l at admission and 117.78 (+/- 38.98) pg/ml after 24 hours of hospitalization.

In foals less than two weeks of age with evidence of SIRS mean serum CGRP concentration was 33.9 (+/- 9.76) pg/ml. Mean serum CGRP concentration was 46.82 (+/- 22.24) pg/ml at admission, 27.26(+/- 13.43) pg/ml after 12 hours of hospitalization and 27.64 (+/- 15.02) pg/ml after 24 hours of hospitalization.

Mean serum CGRP concentration was significantly higher in adult horses with SIRS than healthy adult geldings (see figure 3). Mean serum CGRP concentration was higher in foals with evidence of SIRS than in healthy foals (see figure 4). Serum CGRP concentrations in anesthetized were 13.9 (+/- 8.75 pg/ml) prior to endotoxin exposure and 35.34 (+/- 13.93 pg/ml) 3 hours post endotoxin exposure. Serum CGRP concentration was not significantly higher in anesthetized horses at three hours post exposure to endotoxin than in healthy adult geldings (see figure 5). There was no difference in serum CGRP concentration between healthy adult geldings and healthy foals.

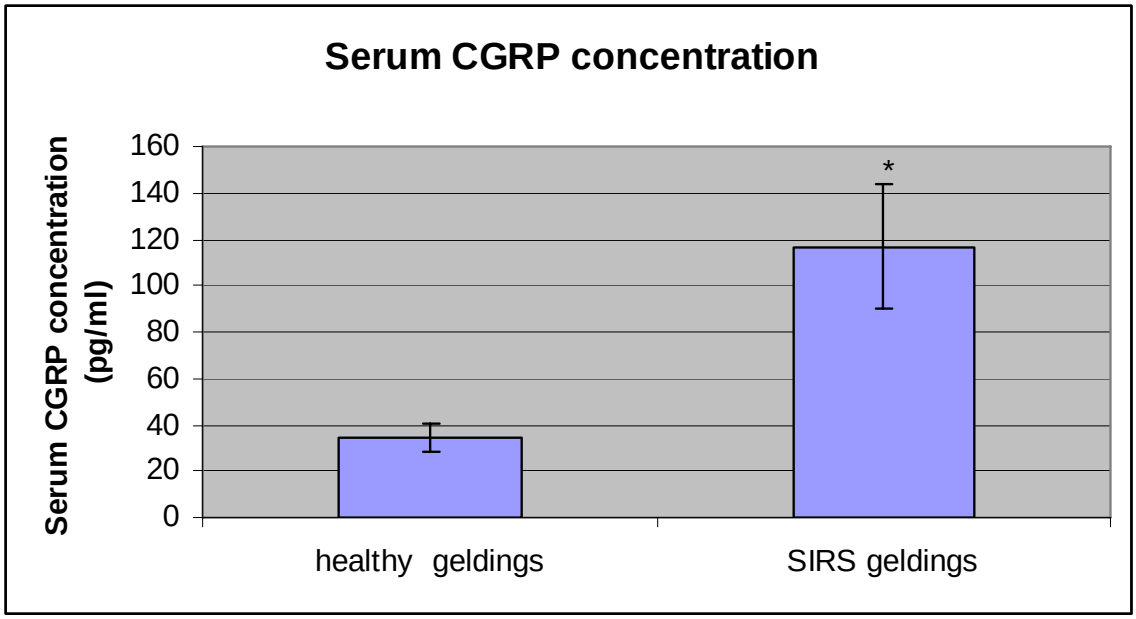

Figure 3: Serum CGRP concentrations in healthy adult geldings and those with evidence of SIRS. * denotes significant difference from healthy geldings

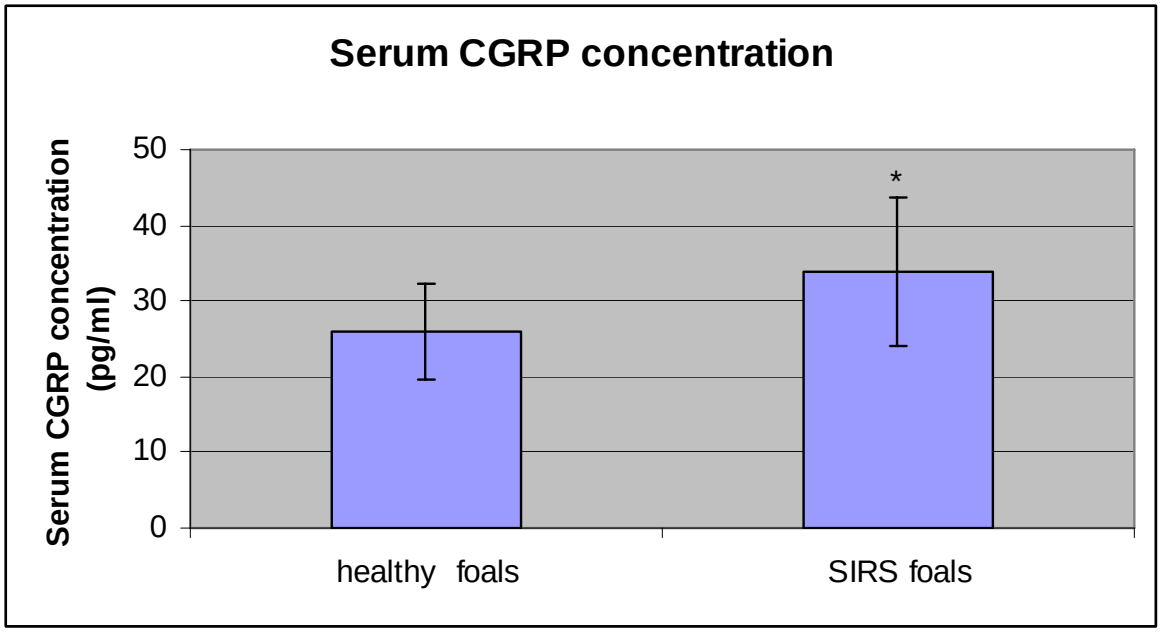

Figure 4: Serum CGRP concentrations in healthy foals and those with evidence of SIRS. * denotes significant difference from healthy foals

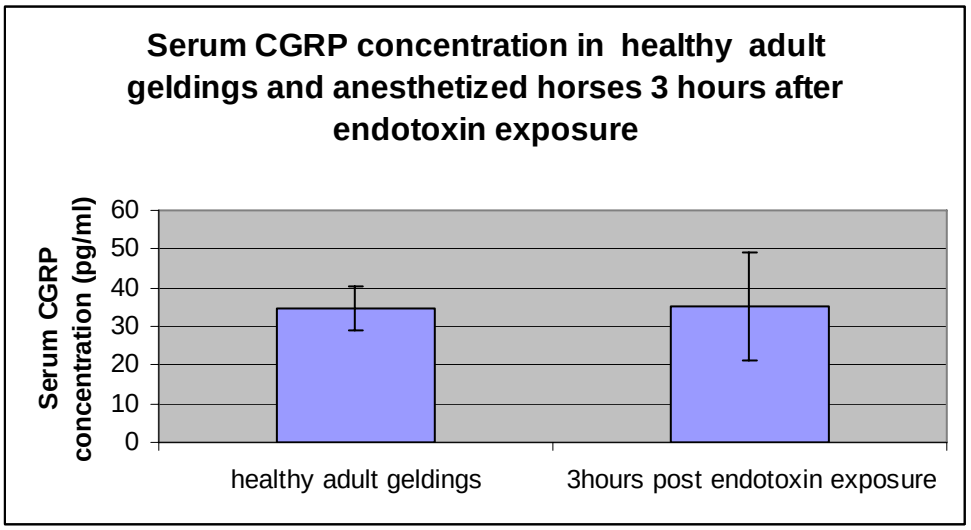

Figure 5: Serum CGRP concentrations in anaesthetized horses exposed to exogenous endotoxin compared to healthy adult geldings.

Discussion

This study showed that equine CGRP could be measured with repeatability using the sandwich ELISA for rat αCGRP. Recovery of CGRP was not 100%, as rat CGRP spiked equine CGRP samples had lower absorbance readings than rat CGRP serum standards. Recovery was 71% from samples with CGRP concentration between 30 and 500pg/ml. The maintenance of parallelism through serial dilutions indicated repeatability of recovery and allowed changes in serum CGRP concentrations with time and relative concentrations between samples to be assessed. Due to the low percentage recovery normal values for serum CGRP in equidae were not established.

In this study healthy mares showed dramatically higher CGRP concentrations at both time points than in any other study group. No difference was noted in serum CGRP concentrations at 8am and 8pm in healthy mares, or in foals with evidence of SIRS during the first 24 hours of hospitalization. This is in contrast to work conducted by Transforini, who found a circadian release of CGRP in healthy human patients (Trasforini *et al.* 1991), where the mean peak plasma CGRP concentration was 18.1 +/- 1.5pmol/l occurring nocturnally, and mean trough CGRP concentration was 11.7 +/- 0.4pmol/l. In patients with systemic disease circadian rhythm was attenuated but not absent (Portaluppi *et al.* 1992).

There was no difference in serum CGRP concentrations between healthy adult geldings and healthy neonatal foals less than two weeks of age of both sexes. While CGRP has important roles in control of placental blood flow and development, little literature evaluates its role in the post partum neonate. CGRP is detectable in serum of term and premature human neonates (Parida *et al.* 1998). Whether this CGRP is produced by the fetus or maternally derived has not been elucidated. Plasma CGRP concentrations have been found to be higher in neonates suffering from polycythemia and are associated with hypocalcemia in these patients (Saggese *et al.* 1992). The authors postulated that CGRP may be implicated in the circulatory adaptation to extra uterine life. How long these factors exist in the post partum period has not been documented. Based on the

almost instantaneous changes in fetal circulation occurring during parturition, it may be postulated that these changes are short lived.

Serum CGRP concentrations were highest in healthy mares. These high serum concentrations were not related to pregnancy and appeared in disagreement with all other data collected in this study and believed to be due to technical issues with the analysis of one ELISA plate . These values were removed from subsequent statistical analysis. Due to the lack of significant differences between any time period for plasma CGRP concentration in foals with evidence of SIRS or in adult horses with evidence of SIRS, this data was grouped prior to statistical analysis. Adult horses with SIRS had higher serum CGRP concentrations than healthy geldings. No differences were noted in CGRP concentrations at 0,12 or 24 hours after hospitalization. This was expected since the time of onset of disease processes culminating in SIRS was variable prior to presentation

Healthy adult geldings had a mean serum CGRP concentration of 34.47 +/- 5.64pg/ml. This is higher than the serum concentrations reported in healthy human patients of 2.0+/- 0.3pg/ml(Joyce *et al.* 1990a), 37pmol/l (14.06pg/ml) (Henriksen *et al.* 2000), 30+/- 26pmol/l (11.4 +/- 9.88pg/ml) (de los Santos and Mazzaferri 1991) and 36.6 +/- 6.2pmol/l (13.9 +/- 2.36 pg/ml) (Schifter *et al.* 1991). In addition, due to the less than 100% recovery using the EIA kit, actual values of equine CGRP concentration were likely higher than those obtained using this assay. Plasma CGRP concentrations in healthy rats are reported as 30.5+/-3.3pg/ml(Griffin *et al.* 1992). This is very similar to the mean serum CGRP concentration reported in this study. In systemic disease serum concentration of CGRP are reported to rise. In patients with confirmed sepsis CGRP serum concentrations were significantly higher than in healthy controls (14.9 +/- 3.2pg/ml vs. 2.0+/- 0.3pg/ml), a rise of 7.5-fold (Joyce *et al.* 1990a). In rats, after experimental induction of endotoxemia plasma CGRP concentration rose to 63.7 +/- 4.6pg/ml, a 2-fold increase(Griffin *et al.* 1992). In this study serum CGRP concentrations in horses presenting with SIRS were 116.92 +/- 26.76pg/ml, a 3-fold increase and statistically significantly different from healthy geldings ($p<0.0002$). The mechanisms

involved in upregulation of CGRP in sepsis have not been elucidated. Given the role of CGRP as a potent vasodilator, it is likely to play a role in the hypotension associated with sepsis.

In term normal birth weight human infants mean plasma CGRP concentrations were 137+/- 17.5 ng/ml (1370 +/- 175 pg/ml), significantly higher than reported values for healthy human adults. In neonates of similar weight with documented systemic illness including sepsis plasma CGRP concentrations were significantly higher at 333.3+/- 40.8ng/ml (3333.3 pg/l +/- 408pg/ml) ($p<0.02$) (Parida *et al.* 1998). This study also reported an age related increase in plasma CGRP and cord plasma CGRP inversely related to degree of prematurity. This shows that fetal and cord plasma CGRP increases in late gestation up to term. Because CGRP is a potent smooth muscle relaxant, an increase in the circulatory concentration of this peptide during pregnancy and a decrease at term suggests a role for circulating CGRP in modulating uterine activity during pregnancy and labor. Infusion of pregnant rats with the CGRP receptor antagonist CGRP(8-37), increased fetal mortality and decreased fetal growth (Gangula *et al.* 2002), suggesting a role for CGRP in fetal maturation. Alterations in expression of CGRP in fetal and placental blood with gestation would therefore be likely. Insufficient data was collected in this study to assess whether serum CGRP concentrations were affected by age within the two foal groups. The absence of significantly increased levels of serum CGRP in healthy neonatal foals, the majority of which presented were sampled at 7 or more days of age, suggests that if serum concentrations of CGRP are elevated relative to adults at parturition, these levels may fall within the first week of life. Foals presented with evidence of SIRS had significantly higher plasma concentrations of CGRP than healthy foals ($p<0.003$). This is in agreement with previously discussed human literature and with the increase found in adult horses with SIRS. It is likely that similar mechanisms of up regulation of expression of CGRP occur in adult horses and neonates.

The time between exposure to endotoxin and increased circulating levels of CGRP occurred after two hours in an experimental rat model (Griffin *et al.* 1992) and a pig model (Arden *et al.* 1994). This increases occurred after doses of 3mg/kg *E.Coli*

endotoxin and 100µg/kg *E.Coli* lipopolysaccharide respectively. The dose of endotoxin used in this study was 20µg/kg. This is a lower dose than reported in other species but produced marked changes in cardiovascular parameters consistent with shock (Pantaleon personal communication). Pre-endotoxin exposure serum CGRP serum concentrations were below the limit of detection of the assay in 10 out of 12 horses. There was no significant difference between serum CGRP concentration after three hours of endotoxin exposure than healthy adult horses($p<0.68$), but a definite trend was present with measurable CGRP concentrations in 6 horses. Increase in numbers of horses in this group would have added statistical power, but was not possible as blood were collected as part of a previous study.

Chapter 5: Conclusions

Concentrations of CGRP can be measured in horse serum using a commercial research ELISA kit for rat αCGRP although a low percentage recovery prevents establishment of a normal physiological range. CGRP concentrations are elevated in equine patients with clinical and clinicopathological evidence of SIRS. Further investigation is required to assess whether elevations of serum CGRP correlate with severity of illness or mortality rate, and to document the time course over which these elevations in serum CGRP occur.

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