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**Neural Regulation of Inflammation and Bleeding**

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Abstract of the Dissertation

**Neural Regulation of Inflammation and Bleeding**

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Recent advances in the field of immunology have revealed an unexpected role for the autonomic nervous system, specifically the vagus nerve, in controlling challenges to homeostasis. This work has reinforced the necessity to study disease within the context of whole-animal physiology, and has identified several approaches to develop novel therapeutic agents for inflammatory disease. While well-defined molecular responses to tissue injury can be modulated to therapeutic advantage, significant morbidity and mortality subsequent to traumatic injury remains a daunting clinical challenge. Application of physiological approaches to management of hemostasis may identify previously unrecognized pathways that can be harnessed to control hemorrhage. To this end, studies were performed to determine whether the vagus nerve controls hemostasis and homeostasis in mice and pigs. Electrical vagus nerve stimulation attenuated systemic inflammatory responses in a porcine model of septic shock, as indicated by significantly reduced circulating levels of tumor necrosis factor and inhibition of systemic coagulopathy, as well an improved mean arterial blood pressure. Vagus nerve stimulation also regulated hemostasis in a porcine model of soft tissue injury, as indicated by decreased bleeding time and total shed blood volume, and increased coagulation factor activity. In a murine model of soft tissue injury, vagus nerve regulation of hemostasis was dependent upon the $\alpha$7 subunit of the nicotinic acetylcholine receptor, and pharmacological agents selective for $\alpha$7 were similarly able to reduce bleeding times in mice. Together, these observations indicate that a previously unrecognized neural pathway mediated via the vagus nerve, and a molecular

mechanism requiring the $\alpha$7 subunit of the acetylcholine receptor, regulates hemostasis in vivo. A greater understanding of cholinergic regulation of hemorrhage may provide new opportunities to develop more effective hemostatic agents.

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# Introduction

## Epidemiology and societal cost of trauma and hemorrhage

Trauma is a serious global health problem, accounting for approximately 1 in 10 deaths worldwide (1). The World Health Organization recently estimated that injuries accounted for the deaths of 5 million people in 2000, which is 9% of global annual mortality. Because nearly 50% of those who die are between 15 and 44 years of age, death due to traumatic injury is the leading cause of life years lost (2): 1.6 million died of all causes of violence (self-inflicted, interpersonal, and war-related) in 2000, accounting for half of trauma mortality; road traffic accounts for the next largest proportion, with approximately 1.3 million deaths in 2000, or 2.1% of overall mortality; an additional 20 to 50 million people are injured in road traffic incidents each year, equating to 12% of the global disease burden from injury in 2000 (3, 4).

In the United States, trauma was the third leading cause of death overall in 2001, and the leading cause of death among those aged 1–44 years (5). There were 179,065 deaths from injury in 2006, for an overall rate of 60 per 100,000 U.S. residents (6). Hemorrhage accounts for 30–40% of trauma deaths, and more than one-third of patients who were found or declared dead at the scene of injury had exsanguinated (7). Bleeding is also the primary cause of early death after trauma center admission, with the majority of exsanguinations occurring during the first 48 hours (8). The U.S. Centers for Disease Control and Prevention estimated that, in 2000, $117 billion was spent on injury-attributable medical care, which amounts to approximately 10% of total U.S. medical expenditures (9). The actual economic cost of injury is much higher, because these figures do not include the cost of life and work years lost secondary to injury or death, and loss of property.

There is a relative paucity of therapeutic options for acute hemorrhage and its pathological sequelae, beyond standard care (tourniquet or direct pressure, resuscitation, surgical correction of wounds, and critical care management of sepsis and multiple organ failure). The wide spectrum of traumatic injuries and severities has made it difficult to show improvements in mortality, arguably the most meaningful endpoint in clinical trials for potential new therapies. Nonetheless, several new therapeutic approaches for acute hemorrhage are undergoing clinical evaluation. For example, antifibrinolytic agents, which are widely used in major surgery to reduce clot degradation, are being tried in trauma patients at risk of significant hemorrhage (10). Fibrin sealants and recombinant factor VIIa (rFVIIa), applied to the wound site rather than



Endnotes can be found in the Literature Cited, beginning on page 104.

delivered systemically, are in various stages of preclinical or clinical development. Fibrin sealants reduce blood loss and the need for blood transfusion in elective surgery by accelerating the formation of the fibrin clot, but the requirement for a dry surface has impeded development for use in trauma patients (11, 12). rFVIIa is approved for use in hemophilia patients, and a recent clinical trial has indicated that it significantly reduced the requirement for red blood cell transfusions in blunt force trauma, with a trend toward improvements in mortality and critical complications without increasing adverse events, including thromboembolism (13). Although rFVIIa reduced the number of red blood cell units required by blunt force trauma victims, the high cost of producing a recombinant protein for clinical use brings into question the feasibility of this approach (14, 15). To date, the most effective and least expensive means of controlling hemorrhage remains the tourniquet, invented in 1718 by French surgeon Jean Louis Petit (16).

Understanding the physiological responses to injury have brought to the fore several promising new therapeutic modalities, but a still greater understanding is required if the morbidity and mortality of injury, and associated costs, are to be reduced. Recent developments in the field of immunology have revealed an unexpected role for the autonomic nervous system, specifically the vagus nerve, in controlling immunological homeostasis (17). These observations have reinforced the necessity to study disease within the context of whole-animal physiology; application of this paradigm to the question of injury may provide new opportunities to identify novel hemostatic agents.

Organs of the immune system receive autonomic innervation, and autonomic neurotransmitters modulate the cellular and molecular effectors of the immune response. Similarly, tissues and organ systems that modulate hemostasis receive autonomic innervation, and autonomic neurotransmitters modulate the cellular and molecular effectors of coagulation. Based on these observations, reviewed below, we hypothesized that the autonomic nervous system via the vagus nerve may influence coagulation and bleeding responses to injury.

## Review of autonomic nervous system

The nervous system is composed of the somatic nervous system, which controls voluntary movements, and the autonomic nervous system, which controls subconscious visceral functions. Two divisions of the autonomic nervous system – the parasympathetic and the sympathetic – continuously regulate basic physiological responses, including heart rate and blood pressure, respiratory rate, gastrointestinal motility, and body temperature. In general, the sympathetic and parasympathetic divisions work in opposition: when one stimulates a particular response, the other typically inhibits it. In the quiescent state, spontaneous activity in autonomic motor neurons establishes autonomic tone and basal organ function. This physiological regulatory activity occurs subconsciously, with the hypothalamus providing general coordination of the autonomic nervous system, and the reticular formation of the brain stem regulating distinct autonomic activities (e.g., pupil size, respiratory rate, and blood pressure). Visceral sensory neurons, which travel along somatic pain fibers, inform the central nervous system (CNS) of chemical changes, stretch and visceral irritation. The majority of visceral functions are modulated reflexively, with the hypothalamus acting as a gatekeeper, allowing less than 1% of ascending information to penetrate higher brain centers (see 18 and references therein for a review).

### Sympathetic system

Preganglionic sympathetic neurons arise from cell bodies within the spinal cord, and pass through white ramus communicantes to reach the paravertebral chain ganglion. From there, synapses give rise to postganglionic neurons that innervate the head, heart, and lungs, modulating the upper eyelids, pupils, salivary glands, and heart and respiratory rate. Other preganglionic fibers pass through the chain ganglion to the aortic plexus, where synapses give rise to postganglionic fibers that innervate the liver, stomach, spleen, kidney, small and large intestines, genitalia, and urinary bladder. One branch of the greater thoracic splanchnic nerve passes through the aortic plexus without forming a synapse, and directly innervates the adrenal glands.

The sympathetic nervous system communicates with its target organs through two neurotransmitters: epinephrine (EPI, also known as adrenaline) and norepinephrine (NE, also known as noradrenaline). Both EPI and NE are monoaminergic catecholamines derived from the amino acids tyrosine and phenylalanine in the same biosynthetic pathway, and are released from postganglionic sympathetic neurons. (Preganglionic

sympathetic neurons use acetylcholine as a neurotransmitter.) The activity of NE is limited by the degradation enzymes monoamine oxidase and catechol-O-methyltransferase. Both EPI and NE exert biological effects at target cells through interaction with adrenergic receptors, G-protein–coupled receptors that transduce intracellular signals via second messengers. These receptors are organized into two groups: alpha, which is further divided into subgroups α1 and α2; and beta, which is further divided into subgroups β1, β2, and β3. While EPI and NE bind with little receptor subgroup specificity, specific biologic responses are elicited by these neurotransmitters based on receptor subtype expression on target cells. In general, ligand binding to α receptors increases intracellular calcium levels and decreases levels of cyclical adenosine monophosphate (cAMP), while activation of β receptors tends to increase intracellular cAMP (see 19 and references therein for a review).

The sympathetic nervous system regulates the “flight or fight” response, the first stage of a general adaptation syndrome to stress or perceived threat. Many of these effects are the result of sympathetic secretion of EPI into the blood stream from the adrenal glands, which are modified ganglia adjacent to the kidneys that synapse with blood vessels rather than post-ganglionic neurons. Circulating EPI can activate systemic physiological reactions that include increased heart rate and blood pressure, inhibition of gastrointestinal activity, constriction of blood vessels to the periphery and internal organs with simultaneous dilation of blood vessels to skeletal muscles, and dilation of the pupils. These same physiological responses are regulated by the sympathetic nervous system during daily activity, in the absence of incipient threat: for example, moments before standing up, the sympathetic nervous system becomes active, and prepares the body for changes in blood pressure and perfusion. The sympathetic nervous system also decreases insulin secretion, increases glucose synthesis and release by the liver and glycogen degradation in skeletal muscle, increases lipolysis and fatty acid release from adipose tissue, and increases the diameter of airway passages.

Parasympathetic system

Parasympathetic regulation of homeostasis is coordinated through 12 pairs of cranial nerves that extend from the brain stem through very long axonal processes nearly all the way to the innervated organs. More than 75% of all parasympathetic activity is carried through cranial nerve 10, the vagus nerve. Two vagus nerve branches (left and right) arise in the dorsal motor nuclei of the medulla oblongata, and pass through the

neck to the thorax along each carotid sheath. In the thorax, both vagus nerves subdivide and pass fibers through the cardiac plexuses to innervate the heart, through the pulmonary plexuses to innervate the lungs, and through the esophageal plexuses. At the esophagus, the left and right vagus nerve branches intermingle, and traverse into the abdomen as anterior and posterior vagal trunks. Once in the abdomen, vagus nerve fibers pass through the aortic plexus before separating into branches that innervate the liver, gallbladder, stomach, small intestine, kidneys, and spleen (20). The vagus nerve contains three fiber types: highly myelinated A fibers, which have low activation thresholds; lightly myelinated B fibers; and unmyelinated C fibers, which have high activation thresholds. The B and C fibers have been implicated in regulation of heart rate; no specific function has been attributed to the vagal A fibers, but our recent results allow the hypothesis that the A fibers may contribute to the regulation of innate immunity in the spleen (17, 21, 22, 23, 24).

Acetylcholine is the neurotransmitter released by all parasympathetic and some sympathetic neurons, but its effects are wide-ranging based largely on the diversity of the receptor subtypes expressed on the target cell in the target organ. Cholinergic receptors are categorized into two types: G-protein–coupled muscarinic receptors and ligand-gated calcium ion channel nicotinic receptors. Muscarinic receptors include five distinct family members ($M_1$-$M_5$), which are generally located at cholinergic neuromuscular or neuroglandular junctions in areas outside the brain; muscarinic receptors are also highly expressed in the brain. Nicotinic receptors have been organized into a heterogeneous family containing 17 known members; most commonly, nicotinic receptors are expressed within the brain. In the periphery, nicotinic receptors are expressed specifically at neuromuscular junctions of the somatic nervous system, and within both sympathetic and parasympathetic ganglia. A previously unrecognized role for the α7 subunit of the nicotinic receptor family recently was identified in the regulation of the innate immune system; no parasympathetic role for α7 had previously been defined outside of the brain (25).

G-protein–coupled muscarinic acetylcholine receptors are encoded by intronless genes that share a high degree of homology across species (26). The ligand-binding domain is highly conserved across all five members of the muscarinic receptor family, which has hampered development of subtype-selective receptor agonists and antagonists (27). Upon ligand binding, muscarinic receptors activate specific ion channels and/or second messenger systems depending on the G-protein subunit to which they are coupled. Muscarinic receptors can influence intracellular

potassium and calcium levels through several pathways that include intermediates such as phospholipase Cβ, adenylate cylcase, and phosphoinositides (28). Other subunits couple with muscarinic receptors to activate kinase-mediated pathways, including Rho, phosphoinositide-3 kinases, non-receptor kinases, and mitogen-activated protein (MAP) kinases (29, 30, 31). In the CNS, muscarinic receptors are highly expressed in the cerebral cortex, the hippocampus, and the striatum, where they have been targets of experimental drugs for diseases such as Alzheimer's disease, schizophrenia, and addiction. The M4 subtype in particular is expressed at high levels in the corpus striatum, where its activation attenuates nociception. In the periphery, neuronal muscarinic receptors are targets of both clinically-approved and experimental therapeutics for overactive bladder, chronic obstructive pulmonary disease, and irritable bowel syndrome. "Non-neuronal" nicotinic receptors are also expressed in cells and tissues that appear to lack cholinergic innervation completely – ovaries, placenta, bladder, keratinocytes, endothelium, and lymphocytes – and play a role in embryonic development and cell motility (see 32 and references therein for a review).

Nicotinic receptors have been organized into a heterogeneous family broadly divided into alpha and non-alpha subtypes. The 10 alpha subunits (α1- α10) have, as a distinguishing characteristic, a disulfide-linked pair of cysteines located at residues 192-193 in the α1 subunit, which are not found in the seven non-alpha subunits (β1-4, γ, ε, δ). The subunits combine into a diverse array of homo- or heteropentamers that are located in both neuronal and non-neuronal tissues. All subunits have a large, glycosylated extracellular domain; four conserved membrane-spanning domains (MI-MIV), with a divergent intracellular domain of varying length between the MIII and MIV loops; and an extracellular C terminus. The most well-characterized nicotinic receptors are those found in muscle, which generally contain two α1 subunits, plus β1, ε, and δ, and can be labeled and inhibited by α-bungarotoxin, a snake venom peptide. Neuronal nicotinic receptors can be further classified by their α-bungarotoxin-binding activity: those that contain α2–4 or α6, in complex with either β2 or β4 subunits (or perhaps α5/β3), do not bind α-bungarotoxin; while α7–α10 do bind and are inhibited by α-bungarotoxin (33, 34).

In humans, α7 is encoded by a gene (*CHRNA7*) that spans at least 75 kb along chromosome 15q13-14; α7 is believed to be the earliest ion channel to have evolved, and subsequent subunits evolved as a result of gene duplications and subsequent divergence. The gene encodes 10 exons, with exon 1 encoding the signal peptide. Sequences within exons

2, 4, and 5 appear to be glycosylated in the mature protein, and potential competitive agonist binding sites map to exons 4, 6, and 7. Exons 7, 8, and 9 encode membrane spanning regions I, II, and III, respectively, while exon 10 encodes membrane spanning region IV. Recent structural analyses indicate that the second α-helical membrane-spanning segment, M2, shapes the lumen of the pore and forms the gate of the closed channel. Ligand binds α7 in the amino terminus near a pair of disulfide-linked cysteines at positions 189–190, at a site distinct and separate from the gated pore (35, 36). mRNA studies of human postmortem brain indicate that α7 is alternatively spliced, and that exon 3 must be excluded in order to remove a stop codon and produce a full-length protein. α7 is expressed in mesocorticolimbic system, hippocampus, neocortex, keratinocytes, endothelium, and several other tissue types, including macrophages (25, 37, 38). A partial duplication of the gene for α7 has recently been identified, which includes exons 5-10 and the intervening intronic sequences. *Dupa7* maps proximal to the full-length gene, and appears to be expressed in-frame with four novel exons**.** The function of the expressed protein is not known (39, 40).

The parasympathetic nervous system provides specialized sensory innervation to several areas of the head and face, including the mouth (lips, palate, gums, tongue, and teeth), eyes, ears (cochlea), and nose. Cranial nerves III, IV, VI, XI, and XIII provide motor innervation to the eyes (including retina, pupil, and intrinsic eye muscles), face (for facial expression), and tongue, and many of the cranial nerves innervate various glands, including the salivary, lacrimal, and submandibular glands. These nerves affect many of the cranial reflexes, including corneal (blinking in response to corneal contact), tympanic (mechanical desensitization to loud noise), auditory (moving eyes and/or head in response to loud noise), vestibulo-ocular (movement of eyes to stabilize field of vision), and light reflexes (contraction of pupils). The vagus nerve (cranial nerve X) provides both sensory and motor innervation to the external ear, diaphragm, tongue, esophagus, respiratory tract, and the visceral organs (including liver, spleen, intestine, gallbladder, urinary bladder and pancreas). Reflexes regulated by the vagus nerve include swallowing, the baroreflex, and the inflammatory reflex (20, 25).

## Review of inflammatory responses to infection

The inflammatory reflex is a recently identified function of the vagus nerve, which both senses and regulates innate immune responses. Infection, ischemia, and injury activate cells of the innate immune system, which release pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and high mobility group box 1 (HMGB1), to activate specific immune responses and enhance wound healing and pathogen clearance (41, 42). Mammals have evolved multiple, redundant mechanisms to confine and control innate immune cell activity and cytokine release, because cytokine dysregulation can lead to chronic local or acute systemic inflammatory diseases, including arthritis, inflammatory bowel disease, septic shock, and severe sepsis (43). The inflammatory reflex is one such mechanism, and studies are underway to both better understand the avenues through which it can be exploited for therapeutic benefit (25).

During infection, invading pathogens release metabolic products that are recognized by cells of the immune system, and activate a series of responses that combat the invader. For example, endotoxin, the lipopolysaccharide (LPS) component of the Gram-negative bacterial cell wall, binds a receptor complex, comprised of Toll-like receptor (TLR)-4, CD14, and MD2, on the surface of immune and endothelial cells, and activates signal transduction cascades that culminate in the release of pro-inflammatory cytokines such as interleukin (IL)-1β, TNF, HMGB1, macrophage colony stimulating factor (MCSF)-1, and macrophage migration inhibitory factor (MIF) (44). These and other cytokines diffuse through tissue and attract to the site of infection additional immune cells, including monocytes, macrophages, neutrophils and lymphocytes. Macrophages and other antigen-presenting cells phagocytose cellular debris and pathogens, and present on their surface antigen complexes (45). As antigen-presenting cells accumulate at the site of injury or infection, increased activity leads to increased cytokine production, which in turn recruits more cells to the site. To limit this cyclic activation of cellular immune responses, stimulated lymphocytes at the site of infection release anti-inflammatory cytokines, such as interleukin- (IL-)10, which act in autocrine and paracrine pathways to inhibit pro-inflammatory cytokine release and suppress the function of antigen presenting cells (46).

Failure to control local inflammatory responses can lead to hypotension, organ ischemia and failure, and even death –clinical signs of severe sepsis, which kills about 30% of the 750,000 patients afflicted each year in the U.S. (47). Robust, uncontrolled localized inflammatory responses can lead to chronic, debilitating diseases such as rheumatoid

arthritis and inflammatory bowel disease. Pro-inflammatory cytokines are proven effective drug targets: for example, monoclonal antibodies against TNF are clinically approved for rheumatoid arthritis and Crohn's disease.

TNF is a necessary and sufficient mediator of acute septic shock syndrome because: 1) it is produced in humans and non-human primates during acute bacterial infection; 2) it causes shock and lethal tissue injury in normal, uninfected animals, and 3) neutralizing monoclonal anti-TNF antibodies prevent septic shock during lethal bacteremia in primates (41, 48, 49). TNF-induced hypotension (shock) is associated with widespread hemorrhagic necrosis in the bowel, inflammatory injury in the kidneys and lungs, and adrenal necrosis; these findings are virtually identical to the pathology of acute septic shock induced, for instance, by overwhelming meningococcemia (49, 50, 51). The kinetics of the TNF response in these models of shock are characteristically early and rapid, peaking within 90 minutes of endotoxin administration (49). Similar kinetics for TNF are observed in humans with acute septic shock syndrome: by the time patients receive emergency medical treatment, TNF levels have returned to baseline, undetectable levels (see (43) and references therein for a review). TNF is required for the complete manifestation of acute septic shock during infection, and it can trigger a downstream cytokine cascade that can amplify and propagate subsequent tissue injury and organ dysfunction. TNF knock-out animals are protected from acute septic shock, and a large and compelling body of work has confirmed that anti-TNF antibodies and other TNF-neutralizing compounds are protective in both small and large animal models of acute septic shock (41, 52). Over the past 25 years, a clear biological picture has emerged, revealing that TNF production is tightly regulated to prevent the development of shock and tissue injury, and to assure that low (beneficial) levels of TNF are produced during a healthy and effective innate immune response to infection (25).

Like TNF, HMGB1 is secreted by macrophages, monocytes, and pituicytes upon stimulation with endotoxin, IL-1β, and TNF (42, 53). Unlike TNF, however, the release of HMGB1 from macrophages is delayed some 12–16 hours after stimulation with endotoxin. HMGB1 activates pro-inflammatory cytokine release from human monocytes, including TNF, IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-6, IL-8, macrophage inflammatory protein- (MIP-)1α, and MIP-1β, but not IL-10 or IL-12 (54). The cytokine activity of HMGB1 maps to the DNA-binding B box domain, which induces TNF release from a murine macrophage cell line (RAW 264.7) and is lethal when injected into BALB/C mice; both B box and full-length HMGB1 increase the permeability of cultured

monolayers of human enterocytes (55, 56). HMGB1 signals through both Toll-like receptor (TLR)-2 and TLR4, though primary cell cultures appear to predominantly signal through TLR4 (57). HMGB1 is sufficient to induce the release of other pro-inflammatory mediators (TNF, IL-1β, and MIP-2), and to induce death in both LPS-sensitive and LPS-resistant mice (42, 58). Full-length HMGB1 and the cytokine domain/DNA binding domain B box induce derangements in intestinal barrier function in mice, leading to increased mucosal permeability and bacterial translocation to mesenteric lymph nodes (56).

In vivo, HMGB1 is detectable only in very low levels (<2 ng/mL) in normal mice and humans. HMGB1 appears in the serum of mice 8 hours after endotoxin infusion (25 ng HMGB1/mL serum), plateaus within 16 hours (300 ng HMGB1/mL serum), and remains elevated for 32 hours or more. Similar but somewhat delayed HMGB1 serum kinetics are also observed in mice subjected to sepsis (59). HMGB1 induces acute inflammatory injury in lung, including neutrophil accumulation, interstitial edema, and protein accumulation in the alveolar space (60). Administration of neutralizing anti-HMGB1 antibodies to infected animals is protective, converting lethality rates in a mouse model of severe sepsis from 75% to as low as 30%. Passive immunization with neutralizing antibodies is protective even when the first antibody dose is administered 24 hours after the onset of peritonitis, a time frame that is consistent with HMGB1 release kinetics, and is clinically meaningful and relevant (59). We have shown that administration of caspase inhibitors to suppress apoptosis during murine sepsis also attenuates serum HMGB1 levels, suggesting that apoptotic cells induce the release HMGB1 (61).

The cytokine theory of disease has enabled the development of an array of novel therapeutic strategies for lethal or debilitating acute and chronic diseases that affect multiple organ systems. Initial interest in TNF as a therapeutic target in severe sepsis waned as studies revealed that its response to infection includes a very rapid kinetic profile, appearing in and then disappearing from the blood within a few hours of immunological challenge. Nonetheless, TNF has proven to be an effective therapeutic target in chronic inflammatory conditions such as rheumatoid arthritis. The identification of HMGB1 as a late-acting mediator of severe sepsis has renewed interest in the pursuit of a cytokine-targeted therapeutic modality for severe sepsis and other inflammatory diseases (62). These and other data accumulated over the last 30 years have demonstrated that dysregulated immune responses can be the basis of disease, and that products of the immune system, particularly cytokines, are effective therapeutic targets.

## Neural regulation of inflammation

Because cytokine dysregulation can lead to chronic local or acute systemic inflammatory diseases, mammals have evolved multiple, redundant mechanisms to confine and control innate immune cell activity and cytokine release (62). The inflammatory reflex is one such physiological mechanism that both monitors and regulates peripheral immune responses to inflammatory stimuli (25). The vagus nerve informs the CNS of developing peripheral infections; the cholinergic anti-inflammatory pathway, the motor arm of the inflammatory reflex, exerts significant anti-inflammatory effects through its neurotransmitter, acetylcholine (25, 62). The vagus nerve is particularly well positioned to interface between the immune and central nervous systems because it innervates the organs that are portals of entry or filters for pathogens and their products (63).

The inflammatory reflex provides several opportunities to develop novel anti-inflammatory strategies based on the vagus nerve and its signaling pathways. For example, electrical stimulation of the vagus nerve attenuates cytokine responses to inflammatory stimuli, including bacterial endotoxin, via the α7 subunit of the nicotinic acetylcholine receptor (64, 65). Nicotinic cholinergic agonists, such as nicotine, bind α7 on the surface of macrophages and other immune cells and activate intracellular signaling cascades that inhibit cytokine release (66). Central vagus nuclei are also potential drug targets, because compounds that penetrate the blood-brain barrier and activate efferent vagus nerve signaling effectively modulate cytokine responses to inflammatory stimuli (67).

Muscarinic acetylcholine receptors in the CNS modulate and integrate vagus nerve regulation of visceral functions (68, 69, 70, 71). Central cholinergic mechanisms may modulate peripheral inflammatory responses (72, 73), but the functional CNS-immune system connection has not been elucidated. We have shown that centrally-administered muscarine or the selective $M_1$ agonist McN-A-343 inhibits endotoxin-induced systemic TNF levels. Likewise, we have shown that elevated brain acetylcholine levels via central administration of methoctramine increase vagus cholinergic outflow, as measured by the high-frequency power component of heart-rate variability and decreased systemic TNF levels during endotoxemia (74, 75, 76, 77). Although immune cells express muscarinic receptors, doses of a muscarine formulation that does not cross the blood–brain barrier do not inhibit systemic TNF release during endotoxemia (74, 78, 79, 80). Furthermore, we have shown that atropine methyl nitrate, a peripheral muscarinic receptor antagonist, does

not abrogate the TNF-suppressing effects of electrical vagus nerve stimulation (74). Thus, central, but not peripheral, muscarinic receptors regulate the inflammatory response during endotoxemia and its inhibition by the cholinergic anti-inflammatory pathway. Activation of central muscarinic cholinergic transmission represents an experimental approach to achieve control over unrestrained systemic inflammation (74).

Vagus nerve stimulation has significant therapeutic potential for the treatment of ongoing systemic inflammation (63). Activation of the cholinergic anti-inflammatory pathway is not dependent upon direct electrical stimulation of the vagus nerve, because mechanical vagus nerve stimulation is sufficient to activate the cholinergic anti-inflammatory pathway to the same degree as electrical stimulation. We have shown that vagus nerve stimulation using a non-invasive, carotid massage technique significantly attenuates serum TNF levels during lethal endotoxemia, and likewise attenuates serum HMGB1 levels during lethal murine sepsis. The anti-inflammatory effects of the cholinergic anti-inflammatory pathway are long lasting, because two days after vagus nerve stimulation, animals do not produce high serum levels of TNF in response to lethal doses of endotoxin. Similarly, macrophage cell cultures are intrinsically down-regulated for over 48 hours following exposure to acetylcholine (81).

The vagus nerve has a very low threshold for activation of the cholinergic anti-inflammatory pathway, suggesting a role for highly myelinated vagus A fibers in the regulation of inflammation. This hypothesis is further supported by the observation that the anti-inflammatory effects of electrical vagus nerve stimulation are dissociable from effects on heart rate, a physiological function attributed to vagus B and C fibers (22, 23, 24, 81). The long lasting anti-inflammatory effects of vagus nerve stimulation indicate that the cholinergic anti-inflammatory pathway possesses a capacity to “educate” the immune system, and suggest that signals from this pathway actively change the phenotype of immune cells.

Macrophages, monocytes, and other cells of the reticuloendothelial system target foreign pathogens in the liver, spleen, and other organs, and coordinate the immediate, early response to circulating microbes and endotoxin (20). During endotoxemia, we have shown that IL-1β and TNF mRNA and protein levels increase significantly in liver and lung, with the highest increases (10-fold or more) in the spleen (82, 83). The spleen is therefore a likely target organ of the cholinergic anti-inflammatory pathway, and a potential site for neural education of the immune system. The spleen receives sympathetic innervation through fibers that originate

in the intermediolateral column of the thoracic spinal cord (84, 85, 86). Parasympathetic innervation of the spleen is coordinated through the vagus nerve, which terminates in synapse-like structures in the celiac-superior mesenteric ganglia (87, 88). Most nerve fibers within the spleen are catecholaminergic; while several other neurotransmitters have been implicated in splenic nerve function, no cholinergic fibers have been identified (89, 90, 91, 92, 93, 94). We have shown that vagus nerve stimulation significantly decreases TNF mRNA levels in the spleen, but not the liver, through a mechanism dependent upon the α7 subunit of the nicotinic acetylcholine receptor. Catecholaminergic nerve fibers co-localize with TNF-producing cells in spleen, and catecholamine depletion attenuates the anti-inflammatory effects of vagus nerve stimulation. Likewise, transection of the common celiac branch of the vagus nerve abrogates the catecholaminergic staining and the TNF-suppressing effects of cervical vagus nerve stimulation in spleen (82, 94). These observations suggest that vagus nerve stimulation induces the release of catecholamines and other neurotransmitters in the spleen, which have been found in close proximity to spleen macrophages (95, 96). The neural connection between the vagus nerve and the spleen can allow for rapid and precise control of systemic cytokine production, and potentially control inflammatory cell trafficking to distant sites (82, 97).

Catecholaminergic, but not cholinergic, nerve fibers co-localize with macrophages in the spleen, and depletion of catecholamines abrogates the anti-inflammatory effects of vagus nerve stimulation. However, the nicotinic acetylcholine receptor α7 subunit is a required component of the cholinergic anti-inflammatory pathway, and α7 knock-out mice are insensitive to the TNF-suppressive effect of vagus nerve activity (65). A potential explanation for the disparity between the potential roles of catecholaminergic and cholinergic nerves may be explained by the anatomic localization of the receptors. The nicotinic acetylcholine receptor subunit α7 is expressed in autonomic ganglia, where it mediates fast synaptic transmission (98, 99). It is possible that acetylcholine released by the vagus nerve acts on α7 expressed in the ganglia of the celiac-superior mesenteric plexus to modulate splenic nerve function. This hypothesis is supported by the observation that electrical stimulation of the cervical vagus nerve decreases pancreatic NE release induced by electrical stimulation of thoracic (sympathetic) nerves, an effect that is independent of muscarinic receptors (100).

Acetylcholine directly down-regulates macrophage cytokine release via the α7 subunit of the nicotinic acetylcholine receptor (82, 101). Likewise, we have shown that the selective α7 agonists nicotine and GTS-

21, an experimental therapeutic for the treatment of Alzheimer's disease, attenuate endotoxin-induced TNF release and lethality through a mechanism dependent upon α7 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (102). The protein complex NF-kB is a transcription factor that modulates cellular responses to stress, and plays a central role in regulating the immunological response to infection (103). Furthermore, the spleen contains and secretes acetylcholine, which may be released by the splenic nerve and subsequently attenuate TNF production (104, 105, 106). Acetylcholine is rapidly degraded outside the synapse by acetylcholine esterase; the resultant short half-life of acetylcholine (~1-2 msec) makes it unlikely that this neurotransmitter diffuses far in tissue (107). However, we have shown that choline, a metabolic byproduct of acetylcholine degradation, is similarly able to attenuate endotoxin-induced TNF release via α7- and NF-κB-dependent mechanisms (108). It is therefore plausible that pre-synaptic vagus nerve signals release acetylcholine, which binds α7 receptors on the post-synaptic catecholaminergic nerve. In the spleen, catecholaminergic signals via the splenic nerve enhance acetylcholine or choline levels in the spleen, which attenuate cytokine production by signaling through α7 on TNF-producing cells.

The cholinergic anti-inflammatory pathway also regulates HMGB1, a pro-inflammatory cytokine that has a significantly delayed kinetic profile as compared with TNF. Transcutaneous vagus nerve stimulation, initiated to coincide with appearance of HMGB1 in the circulation, significantly improves survival and reduces serum HMGB1 levels (81). The nicotinic cholinergic agonists choline and GTS-21 also improve survival during murine sepsis, and attenuate circulating HMGB1 levels (102, 108). We have shown that HMGB1 is a clinically relevant cytokine, because serum HMGB1 levels are elevated in human cerebral and myocardial ischemia (109). In human rheumatoid arthritis, we found significantly elevated serum HMGB1 levels, and a correlation between higher HMGB1 levels and more severe disease. In these patients, we also have shown that vagus nerve activity is significantly lower than in normal control subjects, as evidenced by increased resting heart rate and respiratory rate, as well as decreased high frequency power of heart rate variability, all of which are under vagus nerve control (110, 111, 112). Together these observations indicate that HMGB1 may be under the control of endogenous vagus nerve activity, and that pharmacological agents could be developed in order to target HMGB1 in disease. Furthermore, approaches to increase vagus tone may modulate cytokine release or activity to therapeutic benefit in inflammatory disease.

The identification of the cholinergic anti-inflammatory pathway has expanded the number of experimental approaches that can be used to study CNS regulation of the immune system ("neuroimmunomodulation"), because the vagus is a discrete nerve that can be directly manipulated (e.g., stimulated or severed). Studies of sympathetic regulation of the immune system have been hampered because sympathetic nerves have multiple points of origin in the spinal cord, follow multiple fiber tracts, and innervate numerous organs and tissues, including skin and the vasculature. Moreover, the α- and β-adrenergic receptors can have opposing roles in regulating inflammation, depending on the receptor expressed, the target cell type, and neurotransmitter concentration.

Many organs of the immune system, including liver, thymus, lymph nodes, bone marrow, and spleen, are innervated by sympathetic nerves of ill-defined central origin. The thymus appears to receive sympathetic input from sympathetic chain ganglia that extend from the superior cervical chain ganglia caudal to the T3 sympathetic ganglion, with preganglionic neurons arising in the medulla oblongata, pons, and hypothalamus (113, 114). Sympathetic innervation of lymph nodes has not been well studied; however, the submaxillary lymph node receives sympathetic innervation through the ipsilateral superior cervical ganglion, and association with substance P suggests that lymph nodes may provide an immune sensory function (115, 116, 117). Bones and bone marrow are highly vascularized to provide nutrients to these metabolically-active tissues, and this vasculature receives sympathetic innervation. Sympathetic fibers in the femoral bone marrow arise from the thoracolumbar paravertebral sympathetic ganglia, and T8-L1 spinal sympathetic preganglionic neurons may have origins in premotor sympathetic brain nuclei in the brain stem, pons, and hypothalamus (118). Similarly, the spleen receives sympathetic innervation from preganglionic fibers that arise from the T1-T12 region of the thoracic spinal cord, which synapse at the celiac-mesenteric plexus with the postganglionic fibers that innervate the spleen (85, 86).

The sympathetic nervous system responds to infection and injury with an acute, sustained systemic release of EPI and NE (119, 120). The effects of these neurotransmitters in the bloodstream and tissues are varied, depending on the stimulated cell or tissue type, and the adrenergic receptors expressed (121). Macrophages express both α and β adrenergic receptors, which, in vivo, can stimulate TNF release from naïve macrophages, and suppress TNF release from endotoxin-stimulated macrophages, respectively (122,123). In vivo, generalized activation of the sympathetic nervous system, via stress responses or central injection of inflammatory stimuli, attenuates pro-inflammatory cytokine release in

response to low levels of endotoxin, suggesting that β adrenergic receptors have a dominant effect during minor inflammatory challenge (124, 125). Indeed, electrical stimulation of the splenic nerve induces NE release from the spleen and attenuates LPS-induced TNF through a β-adrenergic-dependent mechanism in the ex vivo perfused rat spleen (126). A vigorous NE response to more robust inflammatory challenges leads to a decompartmentalization syndrome, and spill-over of NE into the bloodstream has untoward, systemic effects on distant tissues and organ systems (127). For example, blood concentrations of NE found during sepsis (~20 nM) induce the release of TNF, IL-1β and IL-6, which in turn cause hepatocellular dysfunction (128, 129, 130). At these concentrations, NE activates $\alpha_2$-adrenergic receptors on Kupffer cells to induce pro-inflammatory cytokine release (131, 132, 133, 134).

These observations indicate that the immune system does not function in near autonomy, as once believed. Like other complex organ systems, the CNS both senses and modulates immune function in real time. Autonomic innervation of the innate immune system, via both sympathetic and parasympathetic pathways, allows for rapid, reflexive, and coordinated regulation of inflammatory responses to infection and injury (17). Increased knowledge about these reflexes provides novel therapeutic targets to control cytokines in inflammatory diseases, and promising approaches include neurotransmitter mimetics, receptor antagonists, and pharmacological and mechanical nerve stimulators. These observations also encourage the application of this approach to the question of injury: identification of reflexes or neural pathways that influence hemostasis may provide similar opportunities to identify novel therapeutic agents for injury and hemorrhage.

## Neural regulation of the vasculature

Neural regulation of blood pressure and perfusion is a fundamental and vital aspect of physiology. For example, systemic blood pressure is detected and modulated through the baroreflex. Baroreceptors within the walls of the aortic arch and internal carotid arteries sense blood pressure, and activate afferent neural pathways to a vasomotor center in the brain stem. In the case of decreasing systemic blood pressure, such as may be experienced during simple postural changes, the vasomotor center responds through efferent sympathetic pathways to induce peripheral vasoconstriction to raise blood pressure (135, 136, 137). Tissue perfusion and blood pressure also can be modulated via changes in the local vasculature through a process known as hyperemia. During increased levels of activity, active hyperemia increases blood flow to an organ or muscle. Reactive hyperemia induces vasodilatation to compensate for reduced oxygen levels and increased metabolic by-products during ischemia. Infection and injury can also induce changes in local perfusion, which facilitate coagulation and pathogen clearance. Sympathetic vasoconstrictor impulses from the brain and vasoactive substances secreted locally by endothelial cells function in concert to invoke local changes in the microcirculation (138).

In supine humans, the cutaneous arterioles receive little neural stimulation, and smooth muscles cells are near basal tone. Environmental stimuli can invoke active vasoconstriction (in response to cold) and reduce blood flow to nearly zero, or can invoke active vasodilatation (in response to heat) and increase local perfusion to as much as 60% of cardiac output (8 L/min) (139, 140). Vasodilatation is regulated through sympathetic cholinergic neurons that terminate at muscarinic receptors, and involves the release of at least one co-transmitter in addition to acetylcholine to induce vasodilatation (141, 142, 143). The most favored hypothetical co-transmitter for this system is vasoactive intestinal polypeptide (VIP), because it activates vasodilatation via cyclic adenosine monophosphate (cAMP); it is found in nerve endings in blood vessels where it co-localizes with acetylcholine; and the peptide $VIP_{10-28}$, which is an antagonist of both VIP type 1 and type 2 receptors, attenuates experimentally-induced vasodilatation (144, 145, 146). However, the role of VIP in active vasodilatation remains controversial, and other agents, including substance P, calcitonin gene-related peptide (CGRP), and histamine, have been hypothesized as vasodilatory co-transmitters (147, 148, 149, 150, 151, 152, 153, 154). Independently of the co-transmitter, it seems likely that acetylcholine mediates vasodilatory effects indirectly, and

activates the synthesis or release of nitric oxide (NO), prostaglandins, and/or endothelium-derived hyperpolarizing factor (EDHF) as an intermediary effector molecule (155, 156). For example, cholinergic stimulation of muscarinic receptors on endothelial cells causes the release of NO, which in turn induces smooth muscle cell relaxation and vasodilatation (157, 158, 159, 160, 161, 162, 163, 164, 165).

The mechanisms that govern active vasoconstriction are nearly as complex as those that regulate vasodilatation. The earliest evidence implicating a role for sympathetic neuroregulation of active vasoconstriction came from observations of increased skin blood flow in patients that had undergone peripheral sympathectomies (142, 166, 167). Classical studies indicate that NE regulates active vasoconstriction through a combination of α1 and α2 receptors, and more recent studies implicate neuropeptide Y and adenosine triphosphate (ATP) as co-transmitters regulating active vasoconstriction (142, 168, 169, 170, 171, 172, 173).

Active neural control of tissue perfusion provides evidence that the vasculature is under reflex control. These reflexes are essential for regular modulation of blood pressure and localized blood flow during changes in posture, temperature, and other common environmental challenges. Many pharmacological agents that target these reflex pathways have been approved for therapeutic use, particularly to regulate hypertension, and to induce controlled hypotension in certain surgical and pathophysiological conditions (e.g., abdominal aortic aneurysm). The role of neural reflexes in the immediate response to injury is undefined.

## Coagulation responses to injury

Blood vessel response to injury includes spontaneous retraction, which exposes to the circulation extravascular tissues, such as vascular smooth muscle cells, injured endothelial cells, basement membrane, and collagen (174). Glycoproteins on the surface of platelets bind exposed collagen, which, together with von Willebrand factor, activates platelets (175, 176). Activated platelets adhere to exposed collagen, and release adenosine diphosphate (ADP) and thromboxane $A_2$, which act in a paracrine fashion to activate other platelets (177, 178, 179, 180). Platelets accumulate at the site of injury and form a loose platelet plug, which temporarily inhibits blood loss until a more rigid clot forms. The platelet plug provides a surface upon which prothrombin is cleaved to form thrombin, which in turn generates fibrin, the main constituent of a blood clot. Expression of phospholipids on the surface of activated platelets also acts as a catalyst for the proteases that generate thrombin (181).

Two cross-reactive enzymatic cascades regulate blood clot formation during traumatic injury; both pathways culminate in the generation of fibrin, the main constituent of a thrombus (Figure 1). A total of nearly 50 different substances are involved in regulating blood coagulation, most of which are produced in the liver, but also in skeletal muscle, endothelium, platelets, and many other cell sources. The contact activation pathway, which is activated by collagen, includes coagulation factors XII, XI, IX, and VIII, while the tissue factor pathway, activated by tissue factor, is mediated through coagulation factor VII. Both pathways converge upon a final common pathway that includes activated factor X, which induces the sequential activation of thrombin and fibrin to form an insoluble clot (20,182).

Cells in the extravascular space, such as smooth muscle cells and basement membrane fibroblasts, express on their surface tissue factor, also known as cluster of differentiation marker 142 (CD142), or coagulation factor III. When vessels are injured, blood in the extravascular space comes in contact with cells expressing surface tissue factor (183,184). Tissue factor is a cellular receptor and co-factor for factor VII; complexed with cell surface phospholipids, calcium and tissue factor, activated factor VII (FVIIa) catalyzes the conversion of factor X to factor Xa via limited proteolysis (185,186, 187, 188, 189, 190). During this “initiation” phase of coagulation, factor Xa generates fento- or nanomolar amounts of thrombin at a relatively low rate. The low levels of thrombin generated during this phase are generally insufficient to form a solid clot, but provide positive feedback into the coagulation pathway by activating

factor V, which is a cofactor for factor X in the proteolytic activation of prothrombin into thrombin, and by activating factor VIII, which activates factor X (191, 192, 193). Importantly, thrombin also activates factor XI, which initiates the contact activation pathway and accelerates thrombin generation and clot deposition (194).

During trauma, collagen exposure to blood triggers the contact activation pathway by forming a complex with factor XII. Activated factor XII (FXIIa) converts factor XI into FXIa; factor XI can also be activated through a positive feedback loop mediated by thrombin itself (194). FXIa activates factor IX, which in turn activates factor X to form FXa. Factor IX forms a complex with factor VIIIa, which is activated from factor VIII by thrombin, as part of another positive feedback loop (195, 196). The resulting intrinsic factor Xase, which forms on the surface of microparticles, platelets, and other cells, activates factor X at a 50–100-fold higher rate than the FVIIa/tissue factor complex in the tissue factor pathway (195, 197, 198, 199, 200). The combination of the contact activation and tissue factor pathways results in two phases of thrombin generation: the tissue factor pathway initially generates initial, low levels of thrombin, which accumulate until sufficient concentrations of thrombin activate the contact activation pathway through several positive feedback loops (194). Thrombin (also known as activated factor II) activates factor XIII and catalyzes the proteolysis of fibrinogen (factor I) to fibrin (factor Ia), which together cross-link to form an insoluble fibrin clot (201, 202, 203, 204, 205).

Multiple mechanisms have evolved to confine and control activation of coagulation, because hemostatic dysregulation is associated with bleeding (e.g., hemophilia), or excessive clotting (e.g., diffuse intravascular coagulation). Two types of blood-borne anti-coagulant mechanisms include a dynamic inhibitory system that is activated by thrombin in a negative feedback loop, as well as constitutively-expressed serine protease inhibitors. The dynamic inhibitory system is triggered by the interaction of thrombin and its cofactor, thrombomodulin, which is constitutively expressed on the vasculature (206, 207). The thrombin/thrombomodulin interaction sequesters activated thrombin from the bloodstream, and the complex also activates protein C, which, together with protein S, attenuates coagulation by the proteolytic degradation and inactivation of FVa and FVIIIa (208, 209, 210, 211, 212). Constitutively expressed serine protease inhibitors include tissue factor pathway inhibitor (TFPI), a dominant negative regulator of the tissue factor pathway; and antithrombin III, which primarily inhibits the contact activation pathway. TFPI, present at relatively low concentrations in

blood, is a stoichiometric inhibitor of FXa and the FVIIa-TF-FXa complex (213, 214, 215). Antithrombin III inhibits all serine proteases produced during the blood coagulation process (216). Together, these anti-coagulant systems restrain clot formation to the site of vascular injury.

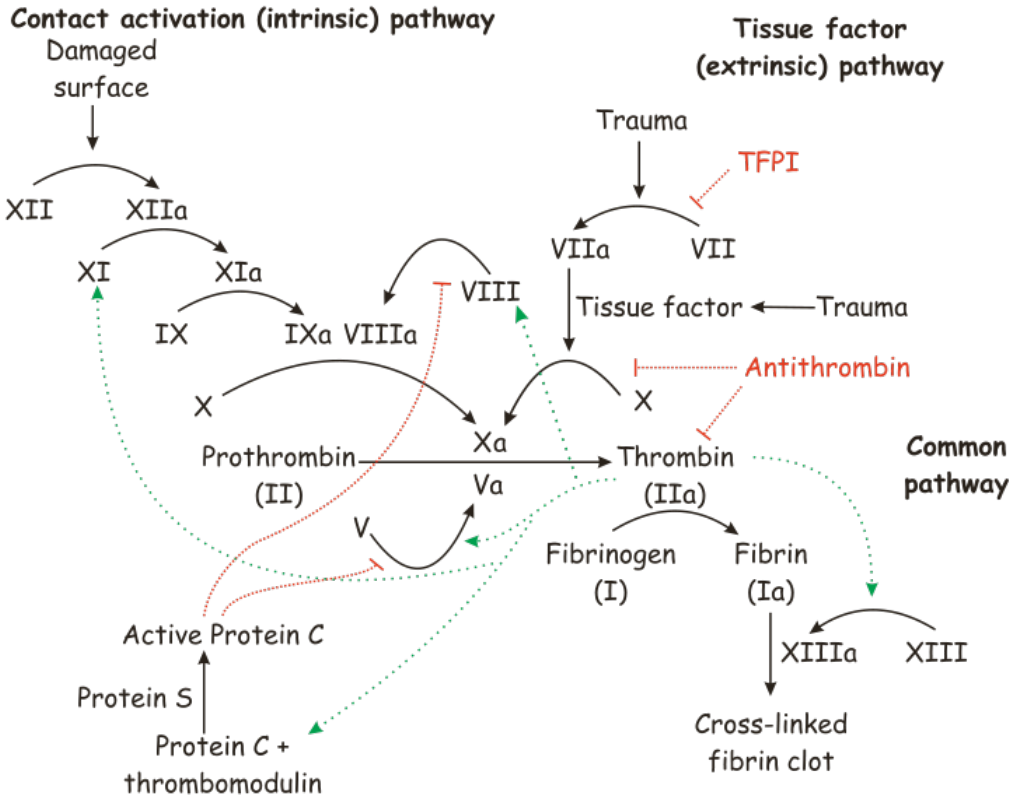
A potential connection between the nervous system and hemostasis has been known for many years. The well-known flight or fight response increases heart rate and blood pressure, while also leading to peripheral vasoconstriction, which together may activate platelets via shear stress. Increased sympathetic activity may activate coagulation via more direct mechanisms, because EPI stimulates platelet aggregation in vitro via α2-adrenergic receptors (217, 218). Elevated circulating levels of catecholamines induced by mental stress, physical activity, or catecholamine infusion increase the activity of clotting factors and platelets in vivo (219, 220, 221, 222, 223). Moreover, EPI infusion induces short-term recruitment of platelets and activated factor VIII, which is stored in Weibel-Palade bodies of endothelial cells (224, 225, 226, 227). In vitro, however, only supra-physiologic concentrations of EPI directly induce platelet aggregation, suggesting that EPI may potentiate platelet aggregation in vivo through other pathways such as ADP, collagen, serotonin, and thrombin (228, 229). NE has a similar affinity for α2 adrenergic receptors on platelets and can induce platelet aggregation in vitro at concentrations similar to those found in humans during periods of stress (230, 231, 232). In addition, β2-adrenergic signaling induces the release of von Willebrand factor multimers from endothelial storage sites into the circulation, which increases the activity of factor VIII (223).

The role of the parasympathetic nervous system, and particularly the vagus nerve, in the regulation of hemostasis is less clear. In studies first published nearly 50 years ago, Kudrjashov and Kalishevska described increased fibrinolysis in rabbit kidneys challenged with thrombin, and noted that this effect was lost following denervation of the organ. The authors also demonstrated that intravenous injection of thrombin led to prolonged coagulation times as a result of increased fibrinolysis in normal animals, but that animals subjected to bilateral cervical vagotomy and challenged with intravenous thrombin died of systemic thrombosis. In contrast, intravenous thrombin led to increased fibrinolysis, and no systemic thrombosis, in animals subjected to bilateral sympathectomy, similar to animals with intact autonomic signaling (233). However, these experiments did not determine whether parasympathetic activity directly modulated fibrinolysis, or if increased sympathetic tone following vagotomy directly stimulated thrombosis (234). Subsequent studies demonstrated that electrical vagus nerve stimulation in both dogs and cats

led to simulatenous increases in thrombosis and fibrinolysis, as a result of thromboplastin (now known to be comprised of tissue factor and phospholipids) and fibrinolytic factors from arterial and venous walls (235, 236, 237, 238, 239, 240, 241, 242). The vagus and other nerves express coagulation activators and inhibitors, including thromboplastin, plasminogen, and plasmin inhibitor, though the activity of these factors is related to nerve signaling or repair following injury (243, 244). Acetylcholine, the parasympathetic neurotransmitter, causes vasodilatation in part by inducing the release of NO, which in turn is a potent antagonist of platelet aggregation (245, 246, 247). In contrast, acetylcholine increases prothrombin gene expression and thrombin activity in cell culture (248). Together, these observations provide evidence that autonomic neurotransmitters are capable of influencing coagulation responses in vivo, though the molecular mechanisms of these effects, and the precise roles of sympathetic and parasympathetic nerves, have not been defined.

**Figure 1: Diagram of the coagulation cascade.**

Two cross-reactive enzymatic cascades regulate blood clot formation during traumatic injury; both pathways culminate in the generation of fibrin, the main constituent of a thrombus. The contact activation pathway, which is activated by collagen, includes coagulation factors XII, XI, IX, and VIII. The tissue factor pathway, activated by tissue factor, is mediated through coagulation factor VII. Both pathways converge upon a final common pathway that includes activated factor X, which induces the sequential activation of thrombin and fibrin to form an insoluble clot. Thrombin provides a positive feedback loop by activating several coagulation factors (green arrows). Negative regulators of coagulation include tissue factor pathway inhibitor (TFPI), antithrombin, and activated protein C, which inhibit the activity of several coagulation factors (red arrows).


Contact activation (intrinsic) pathway
Damaged surface
XII
XIIa
XI
XIa
IX
IXa
VIII
VIIIa
X
Xa
Prothrombin (II)
Thrombin (IIa)
Va
V
Active Protein C
Protein S
Protein C + thrombomodulin
Tissue factor (extrinsic) pathway
Trauma
TFPI
VIIa
VII
Tissue factor
Trauma
Antithrombin
X
Common pathway
Fibrinogen (I)
Fibrin (Ia)
XIIIa
XIII
Cross-linked fibrin clot

## Hypothesis

Immediate responses to physical injury include activation of the fight or flight response, an acute activation of the sympathetic nervous system that increases blood flow, cardiac output, and blood pressure, and heightens the senses. Local vasoconstriction mediated in part by neural reflexes helps slow initial blood loss; this is followed by the activation of platelets and the coagulation cascade. Adrenergic signaling in vascular endothelial cells increases the levels and activity of coagulation factors and platelets in the blood, raising the possibility that neural signaling, originating in the autonomic nervous system, may influence hemostasis. Our discovery of the cholinergic anti-inflammatory pathway highlights the important role of the autonomic nervous system modulating homeostasis, and allows the investigation of reflexive control of coagulation and hemostasis.

In order to test the hypothesis that the autonomic nervous system regulates hemostasis in injury, we developed murine and porcine models of soft tissue injury and tested the effects of electrical vagus nerve stimulation on bleeding time, blood loss, and coagulation parameters. We used these model systems to address our hypothesis in three specific aims:

1. Test whether electrical vagus nerve stimulation can attenuate the pathological sequelae of endotoxemia in swine;
2. Test whether electrical vagus nerve stimulation can attenuate bleeding responses to soft tissue injury in swine and rodents;
3. Test whether cholinergic signaling can recapitulate the hemostatic effects of vagus nerve stimulation.

To determine whether electrical vagus nerve stimulation could influence physiological parameters in pigs as we have observed in mice and humans, we first examined the integrity of the cholinergic anti-inflammatory pathway in pigs. Pigs received electrical vagus nerve stimulation, and were infused with endotoxin; blood was collected and TNF levels, coagulopathy, blood pressure, and requirements for resuscitation were analyzed. The effect of electrical vagus nerve stimulation on hemostasis was tested in pigs in a partial ear resection model, in which bleeding time, shed blood volume, and coagulation parameters were measured following electrical vagus nerve stimulation. Similar endpoints were also measured in a murine partial tail resection model. Finally, to determine whether cholinergic signaling could influence hemostasis, the effects of cholinergic agonists on bleeding time were measured; in addition, the role of the α7 subunit of the acetylcholine

receptor in neural regulation of coagulation was assessed in mice genetically deficient in α7. Together, the results of these studies indicate that the autonomic nervous system, through the vagus nerve, regulates bleeding responses to injury, and that this neural pathway can be activated to reduce bleeding time following injury.

# Methods

## Animals

Adult male 8–12-week-old BALB/c mice (25-30 g, Taconic), and male and female 8-12 week old α7nAChR-deficient mice (C57BL/6 background) and wild-type littermates (Jackson) were housed at 25°C on a 12-h light/dark cycle. Standard animal chow and water were freely available. Mice were euthanized with an overdose of $CO_2$. Adult male 8–12-week-old Lewis rats (250-300 g, Taconic) were housed at 25°C on a 12-h light/dark cycle. Standard animal chow and water were freely available. Rats were euthanized with an overdose of $CO_2$. Piglets of both sexes (25-30 kg) were acquired from a local farm, and allowed free access to food and water prior to the experiment. Animals were euthanized with a bolus intravenous (i.v.) injection of 100 mg/kg pentobarbital followed by 10 mL i.v. KCl (249). All animal experiments were performed in accordance with *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (NIH), under protocols approved by the Institutional Animal Care and Use Committees of The Feinstein Institute for Medical Research (mice and rats) and the city of Vienna (pigs).



Endnotes can be found in the Literature Cited, beginning on page 104.

## Anesthesia

Mice and rats were anesthetized with ketamine (100mg/kg, intramuscularly [i.m.]) and xylazine (10 mg/kg, i.m.). In some experiments, animals were anesthetized with isoflurane (4% induction, 2.5% maintenance via nose cone). Pigs were anesthetized with azaperone (280 mg i.m., STRESNIL®, Jannsen-Cilag, Austria), ketamine hydrochloride (10 mg/kg i.m., KETAVET®, Pharmacia & Upjohn, Germany), and diazepam (10 mg i.m., GEWACALM®, Nycomed, Austria). Thiopental-sodium (100-400 mg, THIOPENTAL®, Biochemie, Austria) was administered through punctured ear veins to maintain anesthesia, as required. Tracheas were intubated and ventilation started at a tidal volume of 10 mL/kg, a respiratory rate of 22/min and a positive end-expiratory pressure level of 2–3 cm $H_2O$. Isoflurane (0.8%) was added to the inspiratory gas ($O_2$, 100%) to maintain anaesthesia. Polyethylene catheters were placed in the right femoral vein and right femoral artery. A 14-Fr. silastic catheter was inserted suprapubically into the urinary bladder. After achieving i.v. access, anesthesia was maintained by continuous infusion of midazolam and sufentanyl. To ensure adequate fluid resuscitation, Ringer's lactate was infused continuously via the femoral vein. Body temperature was measured with a rectal thermometer and maintained at 38-39°C by using a heat blanket (250).

## Disease models

Endotoxemia

As described previously, endotoxin (*Escherichia coli* LPS 026:B6, Difco Laboratories, Detroit, MI) was infused in increasing amounts, beginning with 0.5 to 12 μg/kg/min up to a total dose of 500 μg/kg i.v. endotoxin over a period of 1 h (251). Ringer's solution was infused at a rate of 7 mL/kg i.v. To achieve adequate fluid resuscitation, the infusion rate was increased to 10 mL/kg/h if mean arterial pressure (MAP) fell to <70 mm Hg and to 15 mL/kg/h if MAP was <50 mm Hg. If pulmonary artery occlusion pressure (PAOP) exceeded 10 mm Hg, the rate of infusion was lowered to the previous level. Body temperature was kept constant between 38 and 39°C with a heating blanket. Sham animals (n=3) were treated similarly without receiving endotoxin infusion. The animals were followed thereafter for 5 h.

Hemorrhage

After vagus nerve stimulation, rodent tails were immersed in ~5 mL saline at 37°C for 5 min. Tails were then removed from the solution, 2-3 mm of tail was amputated with a scalpel and returned to the solution (modified from 193, 252). For analyses performed in the absence of anesthesia, mice were placed into 50-mL Falcon tubes, into the base of which holes had been drilled to allow for airflow. The tubes were also shortened so that the tail of a 20–25-g mouse would lie completely outside the tube. As in the anesthetized mice, tails were immersed in ~5 mL saline at 37°C for 5 min. Tails were then removed from the solution, 10 mm of tail was amputated with a scalpel, and the tail was returned to the solution.

To develop a model of peripheral hemorrhage in pigs, a 2-cm-long mark was placed on each hoof with an indelible marker, and then incised with a #11 scalpel to a depth of 1 cm. Blood was allowed to flow freely from the wound site and was collected in a graduated cylinder to measure total blood loss. Bleeding time was measured with a timer; bleeding was defined as having stopped when time between drops exceeded 20 sec. In a second porcine model of peripheral hemorrhage, each ear was warmed to 38+/-0.5°C with a heat lamp for 10 min prior to resection; ear temperature was monitored using a non-contact infrared thermometer. A section 3- cm-wide and 1.5-cm-long was marked with an indelible marker, and then amputated with a #11 scalpel.

A porcine model of non-compressible hemorrhage was developed (250). Briefly, median laparotomy was performed, and standardized

perforating defects were inflicted in the left liver lobe and in the center of the anterior extremity of the spleen, using a 4 cm x 4 cm X-shaped aluminum rod to create a grade IV to V rupture in each organ (according to the Organ Scaling Committee of the American Association for the Surgery of Trauma) (253).

## Vagus stimulation

Mice and rats were placed in the supine position, and a 1–2-cm ventral midline cervical skin incision was made between the mandible and sternum. The subcutaneous tissue and mandibular salivary glands were dissected and retracted laterally. The left vagus nerve was isolated from between the sternomastoid and sternohyoid muscles, dissected free from the neighboring carotid artery, and controlled with a 4-0 silk suture. The nerve was then mounted on bipolar platinum electrodes (Plastics One). Constant current stimuli (1 V or 5 V, 5 Hz, 2 ms pulse width) was applied for 30 sec, 60 sec, 300 sec, or 1200 sec. Electrical stimuli were generated using the STMISOC stimulator adapter, STM100C stimulator module, and MP150 Data Acquisition System from Biopac Systems, Inc. (Goleta, CA). In sham-stimulated animals, the cervical vagus nerve was neither isolated nor dissected free from neighboring structures. A surgical incision was made between the mandible and sternum, and salivary glands and subcutaneous tissues dissected and retracted laterally; the vagus nerve was not manipulated in any way, and electrodes were not placed on the nerve.

In pigs, access to the left cervical vagus nerve was gained through a standard carotid artery cutdown. The vagus nerve was dissected away from the carotid sheath, and lifted slightly with bipolar stimulating electrodes. Electrical stimulation (3.5 mA, 5 Hz, 30 sec) was applied every 5 min for 30 min (total of 7 stimulations). In sham-stimulated animals, the vagus nerve was exposed but not isolated from the carotid sheath or stimulated.

## Pharmacologic agents

Nicotine (Sigma Aldrich) was dissolved in 1X PBS (pH 7.4) to a final concentration of 75 µg/mL, and administered (0.3 mg/kg or 0.03 mg/kg, intraperitoneally [i.p.]) 60 min before tail resection. GTS-21 (Critical Therapeutics, Inc., Lexington, MA) was dissolved in 1X PBS (pH 7.4) to a final concentration of 1 mg/mL, and administered (4 mg/kg or 0.4 mg/kg, i.p.) 60 min before tail resection. Methyllicaconitine (MLA; Sigma Aldrich) was dissolved in 1X PBS (pH 7.4) to a final concentration of 1.5 mg/mL. The MLA group received a single dose of MLA (6 mg/kg, i.p.) administered 20 min before a dose of nicotine.

## Neurectomy

Inferior to the diaphragm, the ventral vagus nerve trunk divides into gastric, hepatic, and celiac branches, and the dorsal vagus trunk divides into gastric and celiac branches (254, 255). The proper branch was visualized, isolated, and severed by making repeated incisions along the splenic artery and vein with a scalpel. In the sham operation, the experimental surgery was replicated with all branches of the vagus left intact and only touched with the tip of a cotton swab, moistened with saline.

## Endpoints

Heart rate

In mice, two unipolar platinum needle electrodes (EL400, Biopac Systems) were inserted subcutaneously into the anterior chest wall. The negative lead was placed in the right midclavicular line at the level of the suprasternal notch, and the positive lead was placed in the left midclavicular line at the level of the xiphoid process. The electrodes were attached to an amplifier (ECG100C, Biopac Systems) that transmitted the recorded signal to a processing unit (MP150, Biopac Systems).

In pigs, mean arterial pressure, mean pulmonary artery pressure, central venous pressure, and intratracheal pressure were continuously monitored using biomedical amplifiers. Heart rate was derived from the electrocardiogram (ECG). All measured signals were transferred to an analog-to-digital converter, displayed on a computer screen, and recorded using a data acquisition system. All monitoring equipment was calibrated before each experiment. Cardiac output was determined by thermodilution technique using a 7.5-Fr Swan-Ganz flow-directed tip catheter (250).

Perfusion (laser Doppler microvascular flow)

To determine the effects of vagus nerve stimulation on microvascular flow, anesthetized mice were subjected to vagus nerve stimulation or a sham stimulation procedure, as described above. Microvascular flow was measured with a TSD144 laser Doppler needle probe attached to an LDF100C amplifier and the MP150 Data Acquisition unit (all from Biopac Systems Inc.). The TSD144 was applied to the surface of the left hindpaw. Data was collected as blood perfusion units (BPU), and backscatter was assessed via tissue remittance (0-100%) to demonstrate stability of Doppler readings.

Bleeding time

Bleeding time was measured with a timer. In mice, bleeding was defined as having stopped when there was no visible blood flow from the wound site for >20 sec. In pigs, bleeding was defined as having stopped when time between drops exceeded 20 sec.

Shed blood volume

In pigs, blood was allowed to flow freely from the wound site and was collected in a graduated cylinder to measure total blood loss.

Cell counts

In anesthetized mice, blood was collected into EDTA via cardiac puncture; platelet counts were performed by Ani-Lytics, Inc. (Gaithersburg, MD). In pigs, citrated blood was collected from the femoral artery catheter or directly from the wound site in 1-mL aliquots. White blood cell counts, red blood cell counts, platelet counts, and hematocrit were analyzed using a Cell-Dyn 1300 (Abbott Diagnostics, Vienna).

Whole blood coagulation

Fifteen µl aliquots of mouse blood were analyzed for activated clotting time (ACT), prothrombin time (PT), and activated partial thromboplastin time (APTT) using the Hemochron Whole Blood Coagulation System (International Technidyne Corporation, Edison, NJ).

Citrated pig blood was collected from a femoral artery catheter; 1 mL aliquots in plastic cuvettes were recalcified with 20 µL of 0.2 M $CaCl_2$ (NATEM solution) and analyzed by rotational thromboelastography (RoTEG; Dynabyte Medical, Munich). Reaction time ("r time"), coagulation time ("k time"), and maximum clot firmness (maximum amplitude, MA) were recorded. Native blood was collected from the wound site, and the same endpoints were analyzed without recalcification. Reaction (r) time measures thrombin generation by the tissue factor pathway; clot formation (k) time measures the high rate of thrombin generation in the contact activation pathway, the full activation of platelets, and formation of a solid thrombus (191, 192, 193, 252, 256, 257).

Platelet aggregation

Citrated pig blood was collected from a femoral artery catheter in 1-mL aliquots and activated with 80 µg of collagen and analyzed by Multiplate (Dynabyte Medical, Munich). Platelet aggregation velocity (AU/min), total platelet aggregation (AU), and area under the curve (AUC; AU*min) were recorded.

TAT ELISA

Blood was collected into EDTA from a femoral artery catheter or directly from the wound site (pigs), or via cardiac puncture (mice), in 1-mL aliquots. Thrombin/anti-thromobin III (TAT) complex ELISAs were performed and calibrated with included standards, according to manufacturer's instructions (Enzygnost TATmicro, Dade Behring Marburg GmbH, Germany). In brief, samples were dispensed into 96-well microtiter plates precoated with anti-TAT antibodies. After washing away unbound substances, horseradish peroxidase-linked anti-TAT antibodies were added. After a wash to remove any unbound antibody-enzyme

reagent, a substrate solution (hydrogen peroxide/tetramethylbenzidine) was added to the wells. The reaction was terminated with a stop buffer and absorbance read at 450 nm using an MRX Revelation (Dynex Technologies, Chantilly, VA) multiwell plate reader. TAT concentration was determined by reference to a standard curve constructed using the human proteins and computer software capable of generating a four-parameter logistic curve fit.

TNF ELISA

Concentrations of TNF were measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. In brief, samples were dispensed into 96-well microtiter plates precoated with monoclonal anti-TNF antibodies. After washing away any unbound substances, horseradish peroxidase-linked monoclonal anti-TNF antibodies were added. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution (hydrogen peroxide/tetramethylbenzidine) was added to the wells. The reaction was terminated with a stop buffer and absorbance read at 450 nm using an MRX Revelation (Dynex Technologies, Chantilly, VA) multiwell plate reader. Concentration of TNF was determined by reference to a standard curve constructed using porcine or murine proteins and computer software capable of generating a four-parameter logistic curve fit.

Illumina gene expression analyses

Total RNA was extracted from mouse spleen using RNeasy Mini Kit (Qiagen, Valencia, CA; Cat# 74104) in accordance with the manufacturer's instructions. Purity and integrity of the RNA preparation was verified by electrophoresis on 1.2% agarose/17% formaldehyde gels. The efficiency of RNA extraction was measured by quantifying absorbance at 260 nm. RNA was biotin-labeled using the EPICENTRE TargetAmp™ Nano-g™ Biotin-aRNA Labeling Kit, according to manufacturer's instructions (EpiCenter, Madison, WI). mRNA expression levels were measured using an Illumina Beadstation and Illumina Mouse 6x chips according to the Illumina Infinium 2 assay manual (Illumina, San Diego, CA, USA), as described previously (258).

Histology of spleen sections

Monoaminergic nerves were visualized in mouse spleens using the sucrose-potassium phosphate-glyoxylic acid (SPG) method of de la Torre (259). Briefly, 10.2 g sucrose, 4.8 g monobasic $KH_2PO_4$ and 1.5 g glyoxylic acid monohydride (Sigma Aldrich) were dissolved in 100 mL

distilled water. NaOH (1 N) was added to adjust to pH 7.4, and the solution was brought to a final volume of 150 mL with distilled water. Organ samples were collected, sliced into 5–10-mm-thick sections, and placed in a pre-cooled cryostat chuck. Once frozen, ~20 µm sections were cut and collected on glass slides. Slides were dipped 3X in SPG and allowed to dry thoroughly. Light mineral oil was placed on each section, and the slides incubated at 95°C for 2.5 min. Slides were visualized under a fluorescent microscope.

## Statistics

Data are presented as mean +/- standard error of measure (SEM), unless indicated otherwise. For all statistical analyses, $p<0.05$ was considered significant.

The Student's T test using the two-tailed, homoscedastic method was performed on all endpoints except as described below.

The effects of vagus nerve stimulation on bleeding time in pigs were analyzed by a mixed models approach to repeated measures analysis of variance (RMANOVA) was used, where the one "between" factor was the grouping factor of vagus nerve stimulation or sham stimulation, and the one "within" or repeated factor was time, i.e., pre- or post-stimulation. The analyses were performed separately on the two endpoints of TAT complex levels at 3 min post-wounding, and at 6 min post-wounding. The results on the untransformed data were not qualitatively different from the transformed data, so for simplicity, the results are displayed in their original units.

The effects of vagus nerve stimulation on TAT concentration were analyzed by first calculating the percent change from the "PreStim" or "PreSham" period to the "PostStim" or "PostSham" period for the endpoints of bleeding time and TAT concentration in blood shed from the wound. The relationship between bleeding time and TAT concentration (in terms of percent change from before stimulation to after) was explored with the Pearson correlation coefficient (the Spearman correlation, a nonparametric counterpart to the Pearson correlation was also computed, and found to be qualitatively the same as the Pearson). A Fisher's z-transformation was performed to compare the two correlations.

# Results

## Pigs have a functional cholinergic anti-inflammatory pathway

Preliminary studies were performed to verify that stimulation of the cervical vagus nerve in pigs could evoke cardiac effects, a well-known response to high levels of vagus stimulation. In initial experiments, animals (n=2) were anesthetized and cervical nerves (left or right vagus nerve) were stimulated with 1-10 mA at 5 Hz, for 180 sec. High-current stimulation of the left or right vagus nerve momentarily stopped heart rate, while stimulation of the sympathetic had a modest stimulatory effect on heart rate and blood pressure. Lower "doses" of current had a less profound effect on heart rate. Notably, even low-power electrical stimulation applied to the right vagus nerve affected both heart rate and blood pressure, whereas lower stimulation parameters applied to the left vagus nerve had no such effect (Figure 2A). To define a clinically-relevant stimulating parameter that did not affect heart rate or blood pressure, anesthetized animals (n=3) received 0–10 mA left vagus nerve stimulation for 30 sec, every 5 min for 30 min. Blood pressure and heart rate were recorded during each 30-sec episode of stimulation; 3 mA was the highest electrical stimulation parameter that did not affect either blood pressure or heart rate (Figure 2B and 2C). These studies indicate that electrical stimulation of the cervical vagus nerves in pigs can activate known vagus nerve–dependent physiological effects, and suggest that this stimulation procedure may also activate the cholinergic anti-inflammatory pathway and the neural tourniquet.

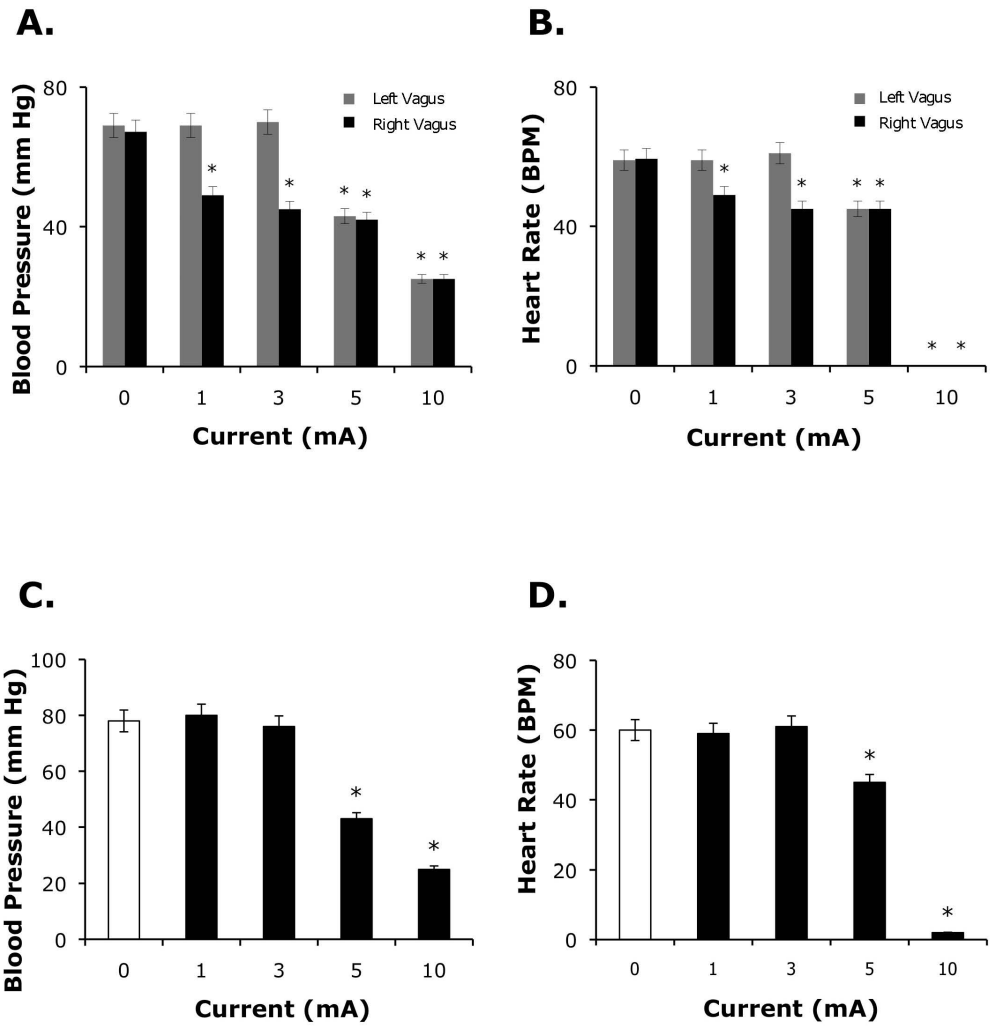


Endnotes can be found in the Literature Cited, beginning on page 104.

**Figure 2: Identification of electrical vagus nerve stimulation parameters that do not affect heart rate or microvascular flow in pigs.**

(A, B) Anesthetized pigs (n=2) were subjected to variable levels of left (grey bars) or right (black bars) cervical vagus nerve stimulation (0-10 mA) for 10 min. Blood pressure (A) and heart rate (B) were measured and recorded once per min for 10 min during vagus nerve stimulation. Blood pressure was recorded as mm Hg; heart rate was recorded as beats per min; each is presented as mean +/- SEM. Student's t-test was used to compare each group to the unstimulated (0 mA) control (*: $p<0.05$).

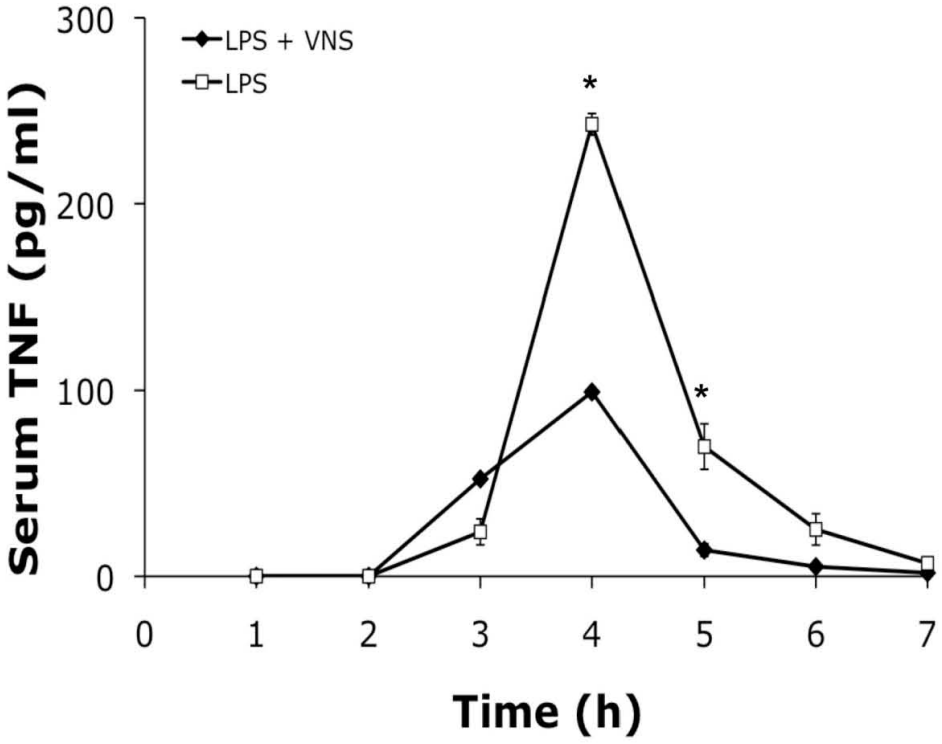
(C, D) Three mA was chosen as a "dose" of electrical vagus nerve stimulation that had no cardiac effects, and was applied to the left vagus nerve of anesthetized pigs (n=3) for 30 sec every 5 min for 30 min, for a total of seven stimulation periods. Blood pressure (C) and heart rate (D) were measured and recorded during each 30 sec period of stimulation. Blood pressure was recorded as mm Hg; heart rate was recorded as beats per min; each is presented as mean +/- SEM. Student's t-test was used to compare each group to the unstimulated (0 mA) control (*: $p<0.05$).


A.
Blood Pressure (mm Hg)
80
40
0
Left Vagus
Right Vagus
0
1
3
5
10
Current (mA)
B.
Heart Rate (BPM)
80
40
0
Left Vagus
Right Vagus
0
1
3
5
10
Current (mA)
C.
Blood Pressure (mm Hg)
100
80
60
40
20
0
0
1
3
5
10
Current (mA)
D.
Heart Rate (BPM)
80
60
40
20
0
0
1
3
5
10
Current (mA)

Studies were performed to verify that stimulation of the cervical vagus nerve in pigs could activate the cholinergic anti-inflammatory pathway and inhibit endotoxin-induced serum TNF levels. Animals (n=3-4/group) were anesthetized as described above, and received left cervical vagus nerve stimulation (VNS; 3.5 mA, 5 Hz) for 30 sec every 5 min for 30 min, beginning 15 min prior to a 1-h infusion of endotoxin (lipopolysaccharide, LPS). Control animals received sham stimulation, in which the cervical vagus was exposed but not stimulated. Blood was collected, and serum prepared and analyzed for tumor necrosis factor (TNF) levels. Vagus nerve stimulation significantly inhibited circulating TNF levels at t=4 h and t=5 h after the beginning of the experiment, as compared with animals that received a sham stimulation procedure ($p<0.05$) (Figure 3). These studies suggest that electrical stimulation of the cervical vagus nerves in pigs can activate the cholinergic anti-inflammatory pathway and attenuate the levels of circulating pro-inflammatory cytokines.

**Figure 3: Electrical vagus nerve stimulation attenuates endotoxin-induced TNF levels.**

Anesthetized pigs (n= 3-4/group) were subjected to left cervical vagus nerve stimulation (VNS; 0 mA, open squares; or 3.5 mA, filled diamonds) for 30 sec every five minutes for 30 min, for a total of seven stimulation epochs; 15 min (t=2h) after completing vagus nerve stimulation, endotoxin (lipopolysaccharide, LPS) infusion began at 0.5 μg/kg/min i.v., and increased logarithmically to 12 μg/kg/min over a period of 1 h, for a total dose of 500 μg/kg. Blood samples were collected 15 min prior (t=1 h) to beginning vagus nerve stimulation, and again every h for 7 h. Serum was prepared from blood samples, and analyzed for tumor necrosis factor (TNF) levels. TNF was recorded in pg of protein/mL of serum, and is presented as mean +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group to the unstimulated control at each time point (*: $p<0.05$).

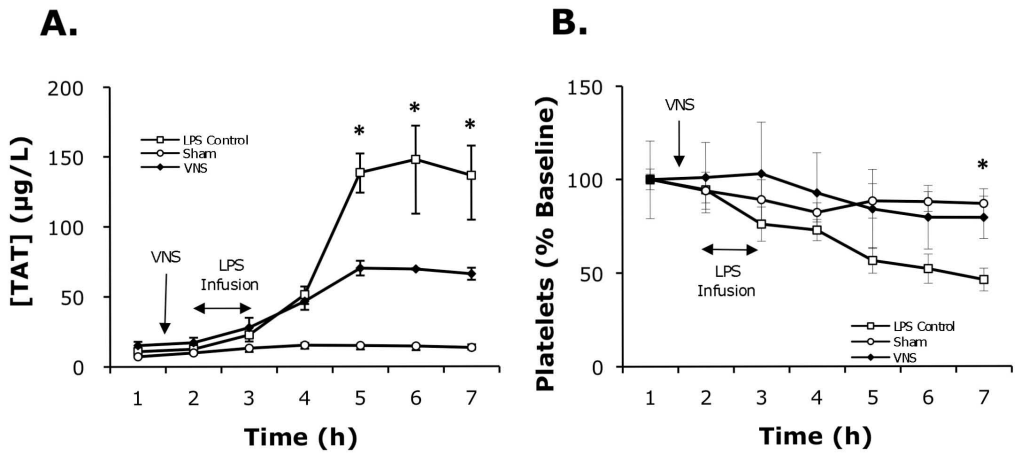

300
200
100
0
LPS + VNS
LPS
*
*
Serum TNF (pg/ml)
0
1
2
3
4
5
6
7
Time (h)

Studies were performed in pigs to determine whether electrical vagus nerve stimulation could inhibit inflammation-induced coagulopathy, a common complication of septic shock and acute sepsis (260). Animals were anesthetized as described above, and received left cervical vagus nerve stimulation (3.5 mA, 5Hz) for 30 sec every 5 min for 30 min, beginning 15 minutes prior to a 1-h infusion of endotoxin. Control animals received sham stimulation, in which the cervical vagus was exposed but not stimulated. Beginning 15 min before electrical vagus nerve stimulation, and once per h thereafter for 7 h, blood was collected and analyzed for thrombin/anti-thrombin complex (TAT) levels, a measure of thrombin production and coagulation status (Figure 4A). At each time point, blood was collected and also analyzed for circulating platelet counts (Figure 4B); depressed circulating platelet numbers are interpreted as an indication of hypercoagulation, because platelets bind to sites of vascular injury and are removed from the circulation (260). Vagus nerve stimulation significantly inhibited circulating TAT levels at the t=5 h, t=6 h and t=7 h time points, as compared with animals that received a sham stimulation procedure ($p<0.05$). Likewise, animals subjected to vagus nerve stimulation had similar circulating platelet counts as compared with control animals that did not receive endotoxin, while animals subjected to endotoxemia without vagus nerve stimulation had markedly reduced circulating platelets. These studies suggest that electrical stimulation of the cervical vagus nerves in pigs can activate the cholinergic anti-inflammatory pathway and attenuate systemic inflammation-induced coagulopathy.

**Figure 4: Electrical vagus nerve stimulation attenuates endotoxin-induced coagulopathy.**

(A) Anesthetized pigs (n= 3-4/group) were subjected to left cervical vagus nerve stimulation (VNS; 0 mA, open squares; or 3.5 mA, filled diamonds) for 30 sec every 5 minutes for 30 min, for a total of seven stimulation epochs; 15 min after the conclusion of vagus nerve stimulation, animals received a 1-h infusion of endotoxin, as described above. Blood was collected 15 min prior to initiating vagus nerve stimulation (t=1 h), 15 min after concluding vagus nerve stimulation (t=2 h), and every h thereafter for a total of 7 h. Serum was prepared and analyzed for thrombin/ anti-thrombin complex (TAT) levels by ELISA. TAT was recorded in μg of protein/L of serum, and is presented as mean +/- SEM. Open circles indicate control animals not subjected to endotoxemia; open squares represent unstimulated endoxemic animals; and filled diamonds represent animals subjected to endotoxemia and vagus nerve stimulation. Student's t-test was used to compare the vagus nerve stimulated group to the unstimulated control at each time point (*: $p<0.05$).

(B) Blood was collected and analyzed on a Cell-Dyn hematometer to determine platelet counts. Platelets were recorded as thousands of cells/mL of blood, and presented as percent change from baseline +/- SEM. Open circles indicate control animals not subjected to endotoxemia; open squares represent unstimulated endoxemic animals; and filled diamonds represent animals subjected to endotoxemia and vagus nerve stimulation. Student's t-test was used to compare the vagus nerve stimulated group to the unstimulated control at each time point (*: $p<0.05$).

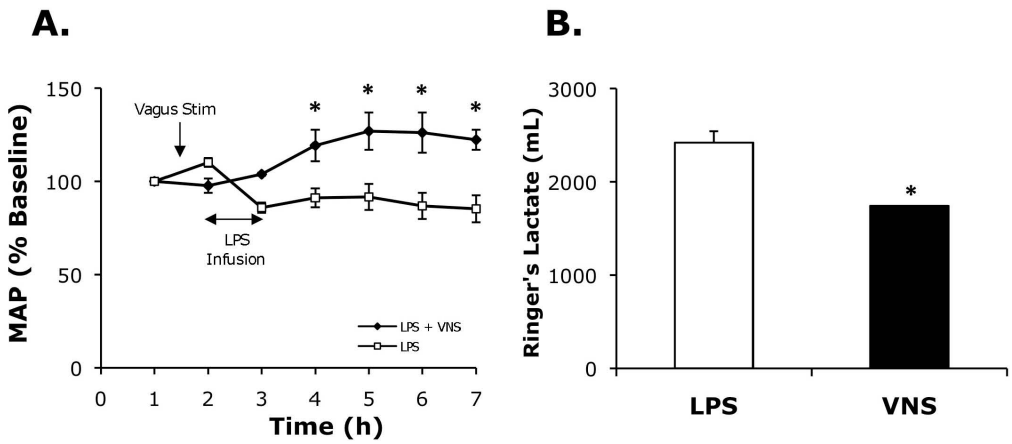

A.
[TAT] (µg/L)
200
150
100
50
0
LPS Control
Sham
VNS
VNS
LPS
Infusion
*
*
*
1
2
3
4
5
6
7
Time (h)
B.
Platelets (% Baseline)
150
100
50
0
VNS
LPS
Infusion
*
LPS Control
Sham
VNS
1
2
3
4
5
6
7
Time (h)

Shock is another common complication of endotoxemia and sepsis. To determine the effect of electrical vagus nerve stimulation on blood pressure during porcine endotoxemia, animals (n=3-4/group) were subjected to electrical vagus nerve stimulation (VNS) and endotoxemia (LPS) as described above. Control animals received sham stimulation, in which the cervical vagus was exposed but not stimulated. Blood pressure increased in animals that received electrical vagus nerve stimulation during endotoxemia, whereas blood pressure decreased in animals that received a sham stimulation procedure (Figure 5A). In agreement with these observations, animals that received vagus nerve stimulation required less resuscitative fluid than animals that received a sham stimulation procedure (Figure 5B). Together, these observations suggest that electrical vagus nerve stimulation may inhibit the development of endotoxemic shock in pigs.

**Figure 5: Electrical vagus nerve stimulation prevents endotoxin-induced decreases in blood pressure.**

(A) Anesthetized pigs (n= 3-4/group) were subjected to left cervical vagus nerve stimulation (0 mA, open squares; or 3.5 mA, filled diamonds) for 30 sec every 5 min for 30 min, for a total of seven stimulation epochs; 15 minutes after the conclusion of vagus nerve stimulation, animals received a 1-h infusion of endotoxin. Mean arterial blood pressure (MAP) was measured and recorded every h, beginning 15 min prior to initiating vagus nerve stimulation (t=1 h), and every h thereafter for a total of 7 h. Blood pressure was recorded as mm Hg, and is presented as percent change from baseline (vs. t=1h) +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group to the unstimulated control at each time point (*: $p<0.05$).

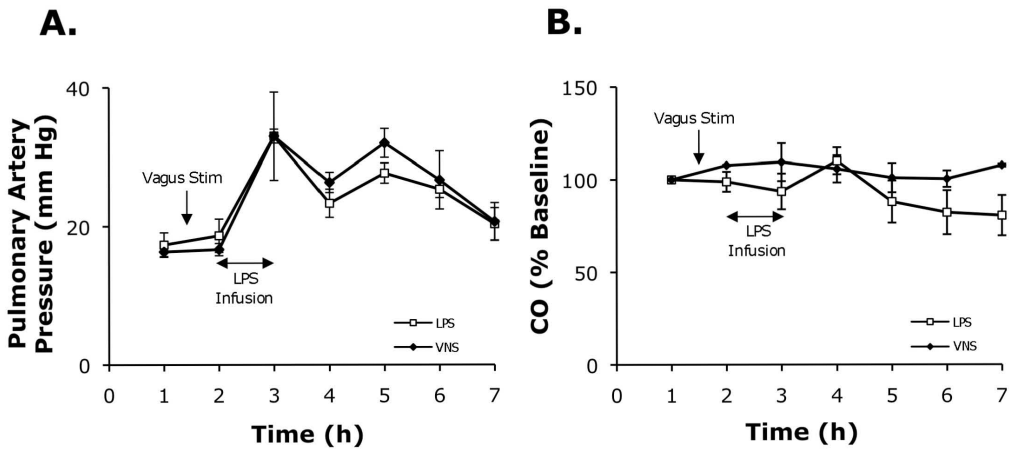
(B) Ringer's lactate was infused throughout the experiment, and titrated according to blood pressure. Total infused Ringer's lactate was recorded in mL, and presented as mean +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group to the unstimulated control (*: $p<0.05$).


A.
B.
MAP (% Baseline)
150
100
50
0
Vagus Stim
LPS
Infusion
LPS + VNS
LPS
0 1 2 3 4 5 6 7
Time (h)
Ringer's Lactate (mL)
3000
2000
1000
0
LPS
VNS

Acute respiratory distress syndrome frequently develops during septic shock and sepsis, and is characterized by increased pulmonary artery pressure and reduced cardiac output (261). To determine the effect of electrical vagus nerve stimulation on cardiopulmonary function during porcine endotoxemia, animals (n=3-4/group) were subjected to electrical vagus nerve stimulation (VNS) and endotoxemia (LPS) as described above. Control animals received sham stimulation, in which the cervical vagus was exposed but not stimulated. Pulmonary artery pressure and cardiac output were monitored throughout the experiment. Electrical vagus nerve stimulation had no significant effect on either parameter (Figure 6).

**Figure 6: Electrical vagus nerve stimulation does not affect cardiopulmonary function during endotoxemia.**

Animals (n=3-4/group) were anesthetized, and the left cervical vagus nerve stimulated (0 mA, open squares; or 3.5 mA, filled diamonds) prior to a 1-h infusion of endotoxin, as described above. Pulmonary artery pressure (A) and cardiac output (B) were monitored throughout the experiment. Values are presented as mean +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group to the unstimulated control at each time point; no statistically significant differences between groups were found.


A.
Pulmonary Artery Pressure (mm Hg)
40
20
0
Vagus Stim
LPS Infusion
LPS
VNS
0 1 2 3 4 5 6 7
Time (h)
B.
CO (% Baseline)
150
100
50
0
Vagus Stim
LPS Infusion
LPS
VNS
0 1 2 3 4 5 6 7
Time (h)

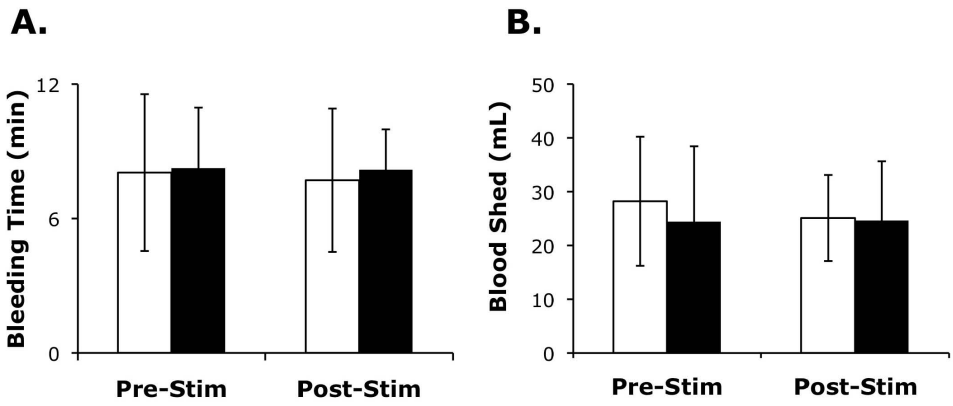
## Vagus nerve stimulation regulates hemostasis in pigs

Having determined that electrical vagus nerve stimulation has biological activity in pigs, we next sought to determine whether this method of activating the vagus nerve could affect the regulation of hemorrhage, as indicated by soft tissue bleeding times. The first step in pursuit of this goal was to develop a reproducible model of hemorrhage. Our first model of peripheral soft tissue injury was based on cuticular incisions, made just above each forelimb hoof. In this peripheral hemorrhage model, bleeding times and shed blood volume were highly variable (Figure 7), in part because the depth and length of the incision, and the time it took to make the incision, were difficult to standardize. We also developed an internal injury model, in which standardized perforating defects were inflicted in the liver and spleen. Although this organ injury model could be readily standardized, our intended primary endpoints, bleeding time and shed blood volume, were difficult to measure (data not shown).

**Figure 7: Cuticle incision is a highly variable model of soft tissue injury and hemorrhage.**

(A) Anesthetized animals (n=4/group) were subjected to uncontrolled peripheral hemorrhage by making a 2-cm incision above the cuticle on the right forelimb. Total bleeding time was recorded; bleeding was defined as having stopped when time between drops exceeded 20 sec.

(B) Shed blood was collected in a graduated cylinder, and total volume recorded. Animals were then subjected to electrical stimulation of the left vagus nerve, and the cuticle incision method was then employed on the contralateral forelimb. Both bleeding time and shed blood volume were expressed as mean +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group to the unstimulated control; no statistically significant differences were found.


A.
Bleeding Time (min)
12
6
0
Pre-Stim
Post-Stim
B.
Blood Shed (mL)
50
40
30
20
10
0
Pre-Stim
Post-Stim

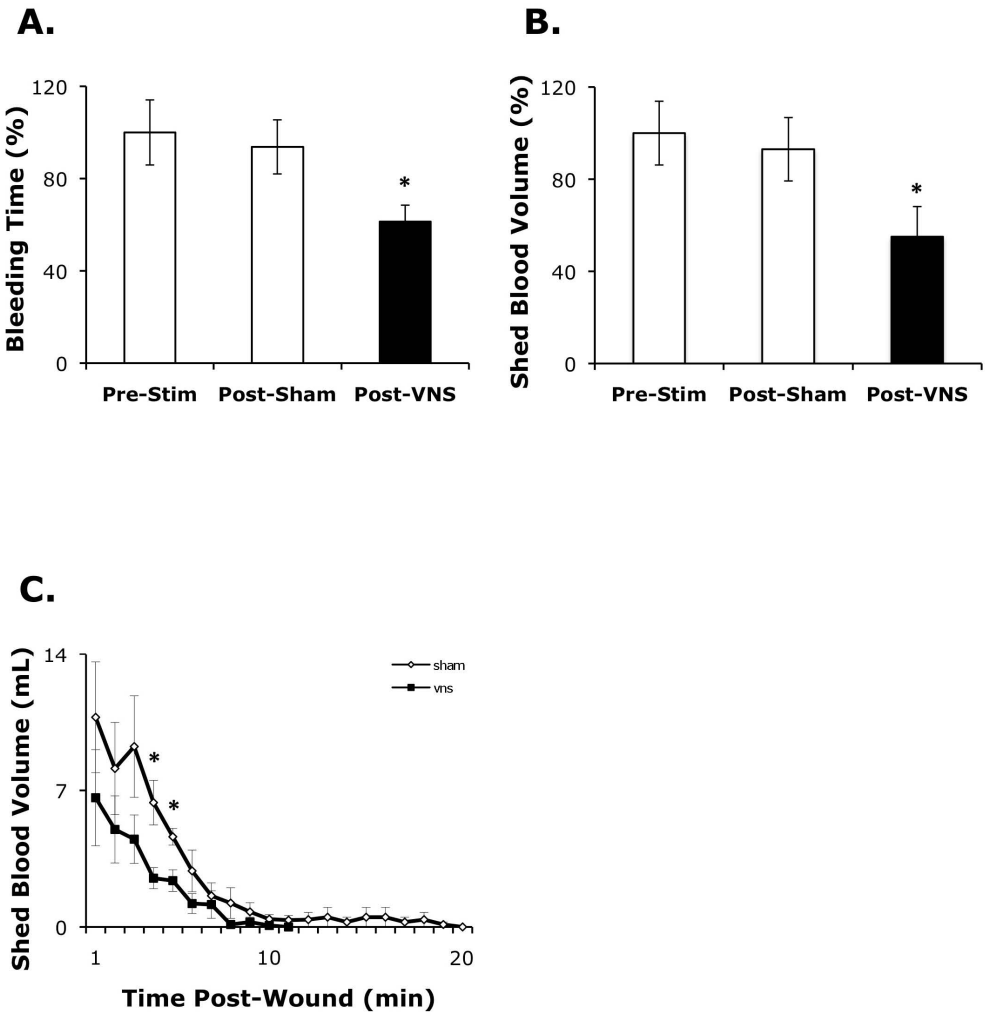
Another peripheral soft tissue injury model, based on partial ear resection, was more easily standardized and gave more consistent bleeding times (Figure 8). Vagus nerve stimulation significantly reduced bleeding time by 40%, as compared with bleeding times from injuries induced prior to vagus nerve stimulation (Figure 8A; *: $p<0.05$). No significant differences in bleeding time were observed after sham stimulation as compared with bleeding time prior to stimulation (bleeding time was reduced by <7% compared with bleeding following the first wounding event) (Figure 8A). Similarly, vagus nerve stimulation significantly reduced the volume of blood shed from the wound by 45% as compared with shed blood volume from injuries induced prior to vagus nerve stimulation (Figure 8B; *: $p<0.05$). No significant differences in shed blood volume were observed after sham stimulation as compared with the volume of blood shed prior to stimulation (shed blood volume was reduced by <8% compared with shed blood volume following the first wounding event) (Figure 8B). To measure the effect of vagus nerve stimulation on bleeding rate, the volume of blood shed from the wounds was measured every minute. The rate of blood loss from wounds following vagus nerve stimulation was lower than blood loss rate from wounds inflicted prior to stimulation, though the effect was significant at only two time points (Figure 8C; *: $p<0.05$). Together, these observations indicate that electrical vagus nerve stimulation attenuates peripheral hemorrhage in pigs.

**Figure 8: Electrical vagus nerve stimulation attenuates peripheral hemorrhage in pigs.**

(A) Anesthetized pigs (n=6-8/group) were subjected to a partial right ear resection. Bleeding began as steady streams of blood, and slowed to a drip over time. Bleeding was defined as having ceased when time between drops exceeded 20 sec. Animals were then subjected to left cervical vagus nerve stimulation, or sham stimulation in which the cervical vagus was exposed but not stimulated; 10 min after vagus nerve stimulation (or sham stimulation), the left ear was subjected to the same partial resection model, and total bleeding time recorded. Bleeding time was recorded in minutes, and is presented as percent of baseline (pre-stimulation bleeding time) +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group (Post-VNS) or the sham stimulated group (Post-Sham) to the unstimulated control (Pre-Stim) (*: $p<0.05$).

(B) Blood shed from the wounds was collected in graduated cylinders and measured in mL. Values are presented as percent of baseline (pre-stimulation bleeding time) +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group (Post-VNS) or the sham stimulated group (Post-Sham) to the unstimulated control (Pre-Stim) (*: $p<0.05$).

(C) The volume of blood shed from the wound was measured every minute until bleeding stopped. Shed blood volume was recorded in mL, and is presented as percent of baseline (pre-stimulation bleeding time) +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group (Post-VNS) or the sham stimulated group (Post-Sham) to the unstimulated control (Pre-Stim) at each time point (*: $p<0.05$).


A.
Bleeding Time (%)
120
80
40
0
*
Pre-Stim
Post-Sham
Post-VNS
B.
Shed Blood Volume (%)
120
80
40
0
*
Pre-Stim
Post-Sham
Post-VNS
C.
Shed Blood Volume (mL)
14
7
0
sham
vns
*
*
1
10
20
Time Post-Wound (min)

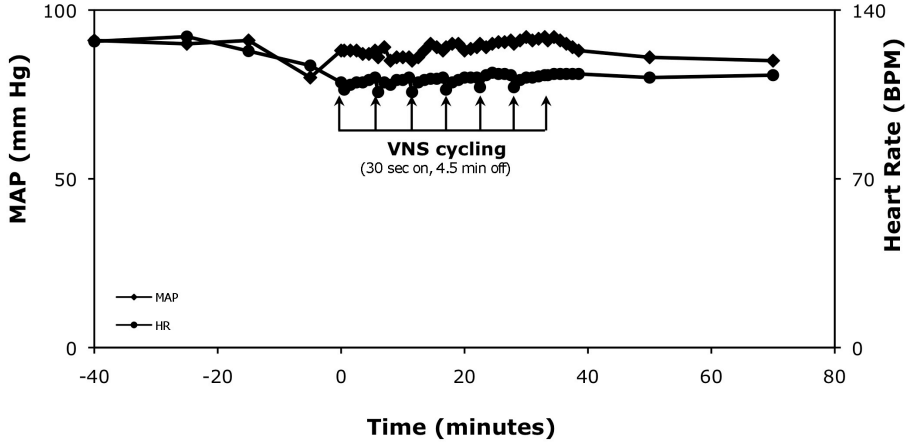
During these experiments, we observed that electrical vagus nerve stimulation occasionally induced a momentary reduction in heart rate and blood pressure. Figure 9A depicts one representative animal; during each 30 sec vagus nerve stimulation epoch, indicated by vertical arrows, momentary decreases in heart rate and blood pressure were observed in some animals. To determine whether maximum observed changes in blood pressure correlated with changes in bleeding times, blood pressure during stimulation was expressed as percent of baseline and plotted against change in bleeding time, also expressed as percent of baseline. Linear regression analysis indicates that blood pressure changes induced by vagus nerve stimulation do not correlate with changes in bleeding time (Figure 9B). These data suggest that another mechanism is responsible for vagus nerve stimulation effects on hemostasis.

**Figure 9: Changes in blood pressure during vagus nerve stimulation do not correlate with changes in bleeding time.**

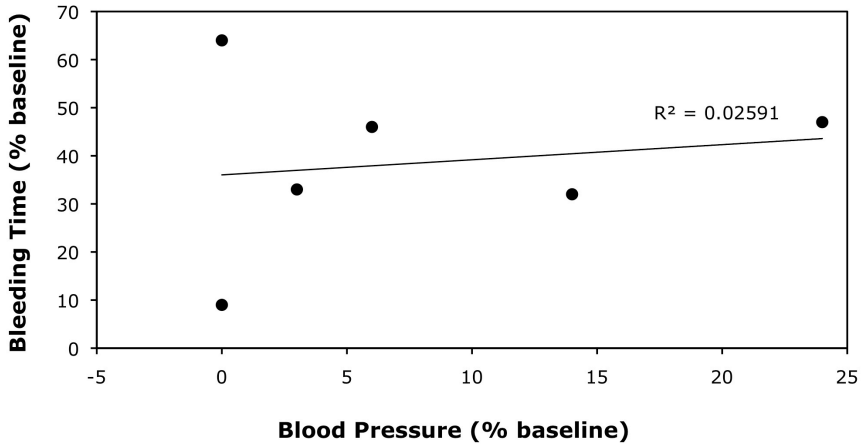
(A) Heart rate (expressed as beats per minute (BPM), and depicted with black diamonds) and mean arterial pressure (MAP, expressed as mm Hg and depicted with black squares) were recorded during the electrical vagus nerve stimulation procedure. Vertical arrows depict each 30 sec epoch of vagus nerve stimulation, over the duration of the 30 minute stimulation procedure (VNS cycling). One representative animal is shown of a total n=6.

(B) Maximal observed changes in blood pressure during electrical vagus nerve stimulation were expressed as percent of baseline, and plotted against change in bleeding time, expressed as percent of bleeding times measured before electrical vagus nerve stimulation. Linear regression analysis was performed to assess the correlation between changes in blood pressure and changes in bleeding time ($R^2$=0.02591).

**A.**


100
50
0
140
70
0
MAP (mm Hg)
Heart Rate (BPM)
VNS cycling
(30 sec on, 4.5 min off)
MAP
HR
-40
-20
0
20
40
60
80
Time (minutes)

**B.**


70
60
50
40
30
20
10
0
Bleeding Time (% baseline)
R² = 0.02591
-5
0
5
10
15
20
25
Blood Pressure (% baseline)

ELISA analysis of shed blood (n=6/group) revealed that vagus nerve stimulation significantly increased the concentration of TAT complex levels in blood shed from the wound site. At 3 minutes post-wounding, the concentration of TAT complex levels in the blood shed from animals receiving sham vagus nerve stimulation had increased 24% from pre-stimulation levels, whereas TAT complex levels in blood shed from animals receiving electrical vagus nerve stimulation had increased >200% from pre-stimulation levels (Figure 10A; *: $p=0.05$). At 6 minutes post-wounding, the concentration of TAT complex levels in the blood shed from animals receiving sham vagus nerve stimulation had increased 49% from pre-stimulation levels (Figure 10B), whereas TAT complex levels in blood shed from animals receiving electrical vagus nerve stimulation had increased >130% from pre-stimulation levels (Figure 10B); this effect on TAT complex levels was not statistically significant. There was a strong positive correlation between changes in bleeding time and changes in TAT complex levels in blood shed from animals subjected to sham stimulation (Figure 10C; Pearson = +0.836; $R^2 = 0.6989$). In contrast, there was a strong negative correlation between changes in bleeding time and changes in TAT complex levels in blood shed from animals subjected to electrical vagus nerve stimulation (Figure 10C; Pearson = -0.919; $R^2 = 0.8447$). The Fischer's z transform revealed that the two correlations are significantly different ($p<0.05$) (Figure 10C).

**Figure 10: Electrical vagus nerve stimulation increases TAT complex levels in shed blood.**

Shed blood was collected in 1-mL aliquots (n=6/group) at 3 min (A) and 6 (B) min post-ear resection, before and after vagus nerve stimulation, and assayed for TAT complex levels by ELISA. TAT levels are presented as percent of baseline (first white bar) in shed blood following electrical vagus nerve stimulation (black bar) or sham stimulation (second white bar), as compared with pre-stimulation TAT concentrations. Values are presented as percent of baseline +/- SEM. RMANOVA was used to compare the vagus nerve stimulated group (Post-VNS) or the sham stimulated group (Post-Sham) to the unstimulated control (Pre-Stim) (*: p=0.05). (C) Changes in TAT complex levels in shed blood were plotted against change in bleeding time. Open circles represent data from sham-stimulated animals; filled circles indicate data from animals that received electrical vagus nerve stimulation. Pearson's correlation coefficient was computed to determine the relationship between bleeding time and TAT complex levels in the vagus nerve-stimulated group (black circles) or the sham-stimulated group (white circles). Fischer's z transform revealed the two correlations are significantly different ($P<0.05$).

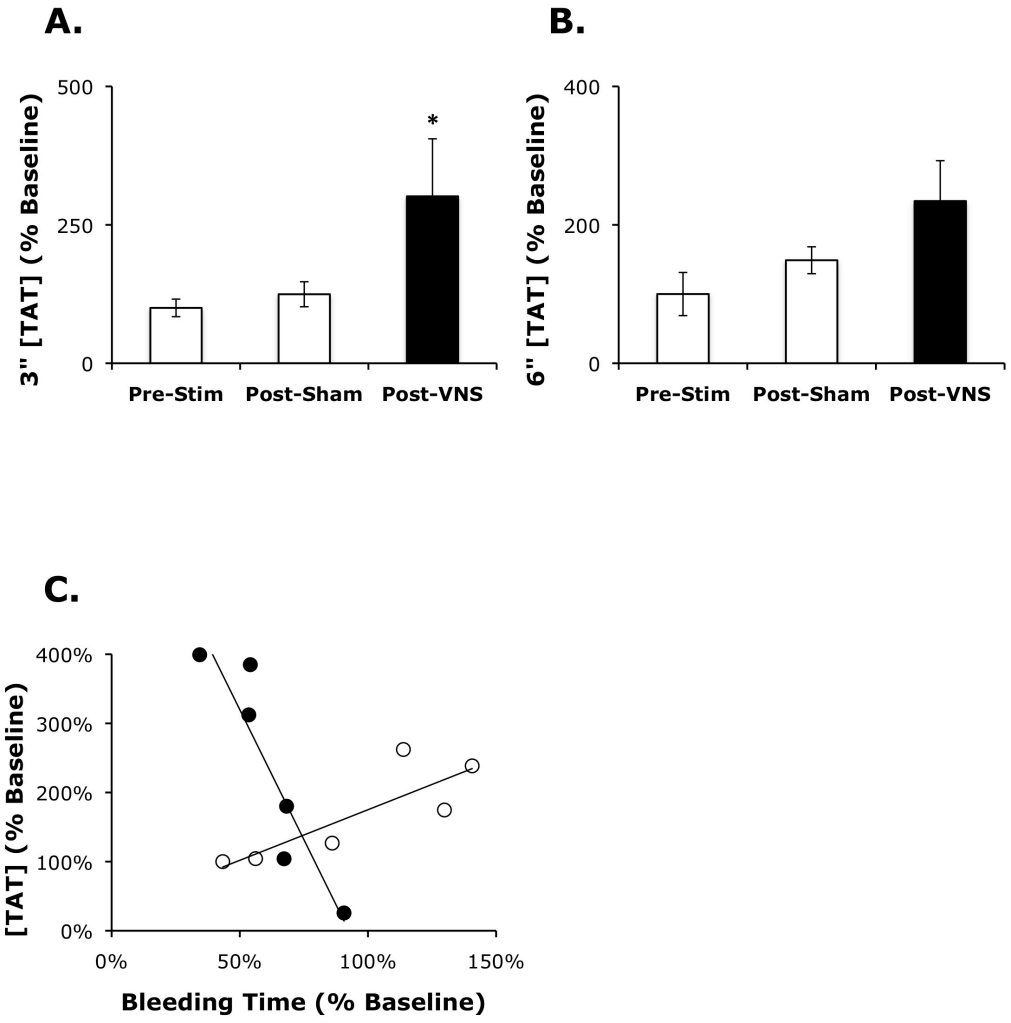

A.
3" [TAT] (% Baseline)
500
250
0
*
Pre-Stim
Post-Sham
Post-VNS
B.
6" [TAT] (% Baseline)
400
200
0
Pre-Stim
Post-Sham
Post-VNS
C.
[TAT] (% Baseline)
400%
300%
200%
100%
0%
0%
50%
100%
150%
Bleeding Time (% Baseline)

Table 1 lists platelet counts and thromboelastography results in shed blood from both sham-stimulated and vagus nerve-stimulated animals (n=2-5/group), at the time of the first ear resection (“Pre-Stim”) and at the time of the second ear resection (“Post-Stim”); no statistically significant changes were observed in blood shed from the wound site. Data presented for these endpoints is for the 3-min post-wounding collection only, as the blood flow rate from wounds following vagus nerve stimulation was too low to allow reproducible measures. Notably, blood flow from the wounds of animals receiving sham stimulation sustained sufficient flow rates to perform RoTEG analyses and obtain platelet counts at 6 min, and in some animals 9 min, post-resection (data not shown).

**Table 1: Local coagulation responses to vagus nerve stimulation.**

| | Post-Sham | | Post-Sham | | Post-Stim | | |
|---|---|---|---|---|---|---|---|
| | Avg. | +/-SE | Avg. | +/-SE | Significance vs. baseline | Avg. | +/-SE | Significance vs. baseline |
| Platelets (k/μL) | 337.7 | 45.6 | 322.0 | 57.4 | 0.82 | 292.0 | 18.1 | 0.57 |
| RoTEG k time (sec) | 517 | 89 | 387 | 44 | 0.21 | 397 | 105 | 0.46 |
| RoTEG r time (sec) | 166 | 29 | 155 | 7 | 0.72 | 130 | 11 | 0.47 |
| RoTEG MA (mm) | 70 | 3 | 70 | 1 | 1.00 | 72 | 2 | 0.65 |

Blood pressure, heart rate, body temperature, hematocrit, red blood cell counts, white blood cell counts, and platelet counts were measured throughout the protocol. Table 2 lists these parameters in both sham-stimulated and vagus nerve-stimulated animals (n=6-8/group), at baseline (prior to surgery) and at the time of the second ear resection ("Post-Stim"). No significant changes were observed in heart rate, mean arterial pressure, or body temperature. Blood collected from an indwelling femoral artery catheter was analyzed for white blood cell count (WBC), red blood cell count (RBC), hematocrit, and platelet count. In animals that received a sham vagus nerve stimulation procedure, red blood cell counts (pre-stimulation = 4.50 +/- 0.10 m/μL vs. post-sham stimulation = 3.65 +/- 0.07 m/μL; $p<0.05$) and hematocrit (pre-stimulation = 25.75 +/- 0.72% vs. post-sham stimulation = 20.84 +/- 0.40%; $p<0.05$) decreased significantly as compared with pre-sham stimulation measurements. In animals that received electrical vagus nerve stimulation, red blood cell counts (pre-stimulation = 4.50 +/- 0.10 m/μL vs. post-sham stimulation = 4.08 +/- 0.17 m/μL; $p<0.05$), but not hematocrit, were reduced significantly. Comparisons between post-sham vagus nerve stimulation measures and post-electrical vagus nerve stimulation measures indicated red blood cell counts (post-sham stimulation = 3.65 +/- 0.07 m/μL vs. post-electrical stimulation = 4.08 +/- 0.14 m/μL; $p<0.05$) and hematocrit (post-sham stimulation = 20.84 +/- 0.40% vs. post-electrical stimulation = 23.02 +/- 1.00%; $p=0.05$) were significantly different.

**Table 2: Systemic responses to vagus nerve stimulation.**

| | Pre-Stim | | Post-Sham | | | Post-Stim | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Average | +/-SE | Average | +/-SE | p= | Average | +/-SE | p= | Significance vs. Pre-Stim |
| MAP | 87 | 2 | 80 | 3 | 0.06 | 87 | 3 | 0.90 | 0.18 |
| Heart Rate | 104 | 6 | 110 | 7 | 0.53 | 103 | 9 | 0.97 | 0.55 |
| Body Temp | 37.8 | 0.1 | 37.9 | 0.1 | 0.55 | 37.9 | 0.2 | 0.56 | 0.93 |
| WBC (k/µL) | 15.3 | 0.9 | 12.9 | 1.2 | 0.14 | 12.5 | 1.1 | 0.13 | 0.84 |
| RBC (m/µL) | 4.5 | 0.1 | 3.7 | 0.1 | 0.00 | 4.1 | 0.1 | 0.04 | 0.02 |
| Hematocrit (%) | 25.8 | 0.7 | 20.8 | 0.4 | 0.00 | 23.0 | 1.0 | 0.07 | 0.05 |
| Platelet Count (k/µL) | 344.9 | 24.6 | 292.7 | 32.6 | 0.22 | 290.0 | 13.2 | 0.22 | 0.95 |

Blood was collected from an indwelling femoral artery catheter and analyzed for whole blood coagulation activity as assessed by rotational thromboelastography (RoTEG); platelet aggregation activity as assessed by the Multiplate analyzer; and TAT complex levels as assessed by ELISA. Table 3 lists the results of these analyses in circulating blood before vagus nerve stimulation, and results in circulating blood following electrical or sham vagus nerve stimulation (n=6-8/group). In animals that received a sham vagus nerve stimulation procedure, platelet aggregation decreased significantly, as compared with pre-sham stimulation samples (pre-stimulation = 55.22 +/- 3.84 AU vs. post-sham stimulation = 42.46 +/- 3.66 AU; $p=0.05$). Platelet aggregation measures were not significantly affected in animals receiving electrical vagus nerve stimulation, but RoTEG analyses indicated that reaction (r) time of circulating blood was significantly reduced as compared with pre-stimulation measures (pre-stimulation = 473 +/- 14 sec vs. post-stimulation = 417 +/- 21 sec; $p<0.05$).

**Table 3: Systemic coagulation responses to vagus nerve stimulation.**

| | | Pre-Stim | | Post-Sham | | | Post-Stim | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Avg. | +/-SE | Avg. | +/-SE | p= | Avg. | +/-SE | p= |
| RoTEG | r time | 473 | 14 | 429 | 26 | 0.12 | 417 | 22 | 0.04 |
| | K time | 135 | 8 | 136 | 10 | 0.93 | 133 | 11 | 0.90 |
| | MA | 64 | 1 | 63 | 1 | 0.64 | 63 | 3 | 0.73 |
| Platelet Activity | Aggregation | 55.2 | 3.8 | 42.5 | 3.7 | 0.05 | 42.6 | 5.5 | 0.09 |
| | Velocity | 10.62 | 0.70 | 8.21 | 1.02 | 0.07 | 8.18 | 0.98 | 0.07 |
| | AUC | 344.38 | 25.67 | 263.00 | 24.21 | 0.06 | 260.33 | 35.37 | 0.09 |
| TAT Complex | TAT (µg/mL) | 10.2 | 2.3 | 14.0 | 0.6 | 0.24 | 13.8 | 1.3 | 0.35 |

## Vagus nerve stimulation attenuates bleeding time in mice

Experiments in porcine hemorrhage models indicated that electrical vagus nerve stimulation modulates hemostasis. In order to explore further the physiological mechanism of this observation, we sought to establish a rodent model of peripheral hemorrhage. We reasoned that a model system in mice would be unlikely to yield sufficiently large blood samples from the wound for molecular analysis. Accordingly, we attempted to develop a peripheral hemorrhage model using anesthetized rats, based on partial tail resection. Initial experiments to identify an appropriate length of tail to amputate (2–10 mm) revealed highly variable bleeding times (data not shown), the intended primary endpoint in the planned experiments. In contrast, preliminary experiments revealed that the same tail resection technique applied to mice provided consistent bleeding times (data not shown).

A primary function of the vagus nerve is to regulate heart rate, which can have a profound affect on peripheral blood flow. To identify vagus nerve stimulation parameters that would not affect heart rate, anesthetized mice were subjected to increasing levels of electrical vagus nerve stimulation for 20 min, and heart rate measured. Stimulation of the cervical vagus nerve with higher electrical parameters (5 V, 30 Hz) had profound effects on heart rate, whereas low electrical stimulation parameters (1 V, 5 Hz) had no measurable effect on heart rate (Figure 11A). To rule out the possibility that electrical vagus nerve stimulation might affect microvascular blood flow without affecting heart rate, laser Doppler studies were performed on the hindpaw. Complete occlusion of the femoral artery with a clamp completely abrogated measurable microvascular blood flow (blood perfusion units, BPU) in the right hindpaw; electrical stimulation of the cervical vagus nerve had a similar effect (Figure 11B). Closer analysis indicated that vagus nerve stimulation-induced decreases in heart rate (EKG) correlate with reduced hindpaw microvascular flow (BPU) (Figure 11C). In contrast, low electrical vagus nerve stimulation parameters had no measurable effect on heart rate (EKG) or microvascular flow (BPU) (Figure 11D). Note that microvascular flow and heart rate returned to normal during extended periods of high-power electrical vagus nerve stimulation (Figure 11D, left panel).

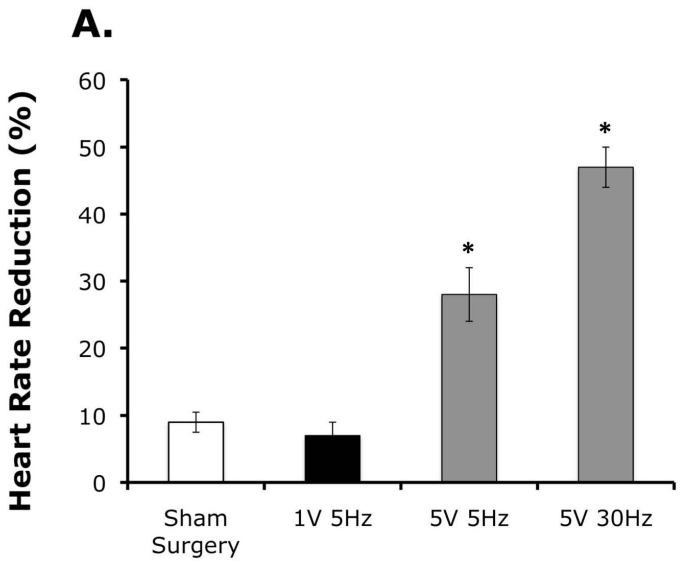
**Figure 11: Identification of electrical vagus nerve stimulation parameters that do not affect heart rate or microvascular flow in mice.**

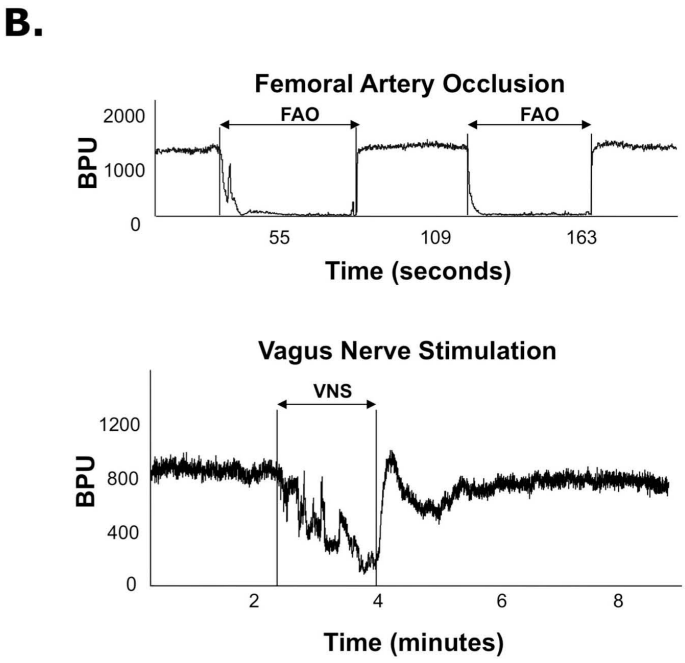
(A) BALB/c mice (n=5/group) received 20 min of electrical stimulation (1 V, 5 Hz [black bar], 5 V, 5 Hz [grey bar], or 5 V, 30 Hz [grey bar]; n=10/group) to the carotid vagus, or sham stimulation (surgery to expose carotid sheath, but without dissection of the nerve from the carotid artery [white bar]; n=10), and heart rate was measured. Heart rate was recorded as beats per minute, and is presented as percent change from baseline (pre-stimulation heart rate) +/- SEM. Student's t-test was used to compare the vagus nerve stimulated groups or the sham stimulated group to baseline heart rate (*: $p<0.05$).

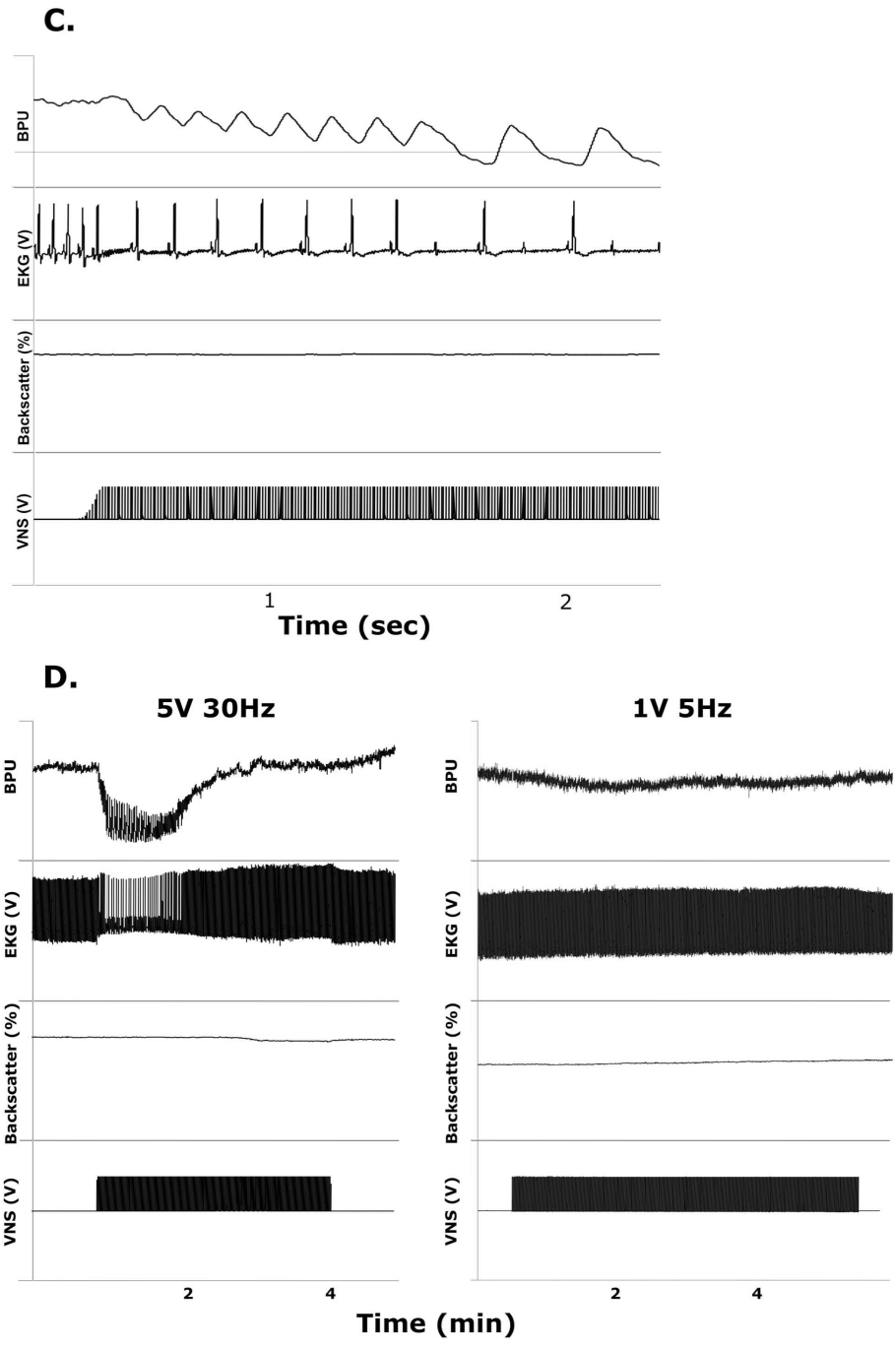
(B) BALB/c mice were subjected to femoral artery occlusion (top panel) or high electrical vagus nerve stimulation parameters (5V, 30Hz) for 120 sec (bottom panel). Microvascular flow (measured in blood perfusion units, BPU) in the right hindpaw foot pad was measured with laser Doppler; representative tracings shown of n=3/group.

(C) Hindpaw microvascular flow (BPU; top panel) and heart rate (EKG; second panel) during the first 2 sec of vagus nerve stimulation (VNS; bottom panel). Also shown is backscatter, a control for BPU (third panel). Representative tracings shown of n=3/group.

(D) Comparison between the effects of high (5 V, 30 Hz; left panel) and low (1 V, 5 Hz; right panel) electrical vagus nerve stimulation parameters on microvascular flow (BPU) and heart rate (EKG). Also shown is backscatter, a control for BPU; and the duration of vagus nerve stimulation (VNS). Representative tracings shown of n=3/group.


A.
Heart Rate Reduction (%)
60
50
40
30
20
10
0
*
*
Sham Surgery
1V 5Hz
5V 5Hz
5V 30Hz


B.
Femoral Artery Occlusion
FAO
FAO
BPU
2000
1000
0
55
109
163
Time (seconds)
Vagus Nerve Stimulation
VNS
BPU
1200
800
400
0
2
4
6
8
Time (minutes)

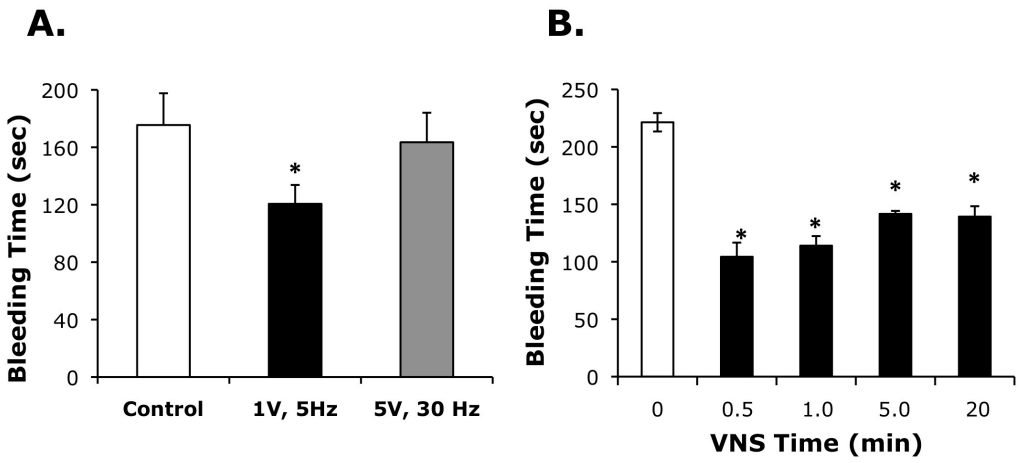

C.
BPU
EKG (V)
Backscatter (%)
VNS (V)
1
2
Time (sec)
D.
5V 30Hz
1V 5Hz
BPU
EKG (V)
Backscatter (%)
VNS (V)
2
4
2
4
Time (min)

Vagus nerve stimulation parameters that did not affect heart rate or peripheral microvascular flow were used to determine the effects on peripheral bleeding times. First, mice received sham vagus nerve stimulation or 20 min of electrical vagus nerve stimulation at 1 V, 5 Hz or 5 V, 30 Hz, and were then subjected to a standard model of tail resection. Vagus nerve stimulation at 1 V, 5 Hz significantly attenuated peripheral bleeding time (sham stimulation = 176+/-22 sec vs. 1 V, 5 Hz stimulation = 121+/-13 sec; $p<0.001$), whereas higher electrical stimulation parameters had no significant effect (sham stimulation = 176+/-22 sec vs. 5 V, 30 Hz stimulation = 164+/-21 sec) (Figure 12A). Next, the effects of different durations of vagus nerve stimulation on bleeding time were studied. All four stimulation durations significantly reduced bleeding time (sham stimulation = 221+/-8 sec vs. 30 sec stimulation = 104.3+/-12.3 sec, $p<0.01$; vs. 60 sec stimulation = 114 +/- 8.3 sec, $p<0.01$; vs. 300 sec stimulation = 141.7+/-2.6 sec, $p<0.01$) (Figure 12B).

**Figure 12: Vagus nerve stimulation reduces bleeding time in mice.**

(A) BALB/c mice received 20 min of electrical stimulation (1 V, 5 Hz [black bar] or 5 V, 30 Hz [grey bar]; n=10/group) to the carotid vagus, or sham stimulation (surgery to expose carotid sheath, but without dissection of the nerve from the carotid artery [white bar]; n=10). Tails were incubated in 37°C saline for 5 min, and removed from the saline; 2 mm of tail was removed with a sharp razor blade, and the tail then re-submerged in the 37°C saline bath. Total bleeding time measured with a stopwatch, and recorded in seconds; values are presented as mean +/- SEM. Heart rate was recorded as beats per minute, and is presented as percent change from baseline (pre-stimulation heart rate) +/- SEM. Student's t-test was used to compare the vagus nerve-stimulated groups to the sham-stimulated group (*: $p<0.05$).

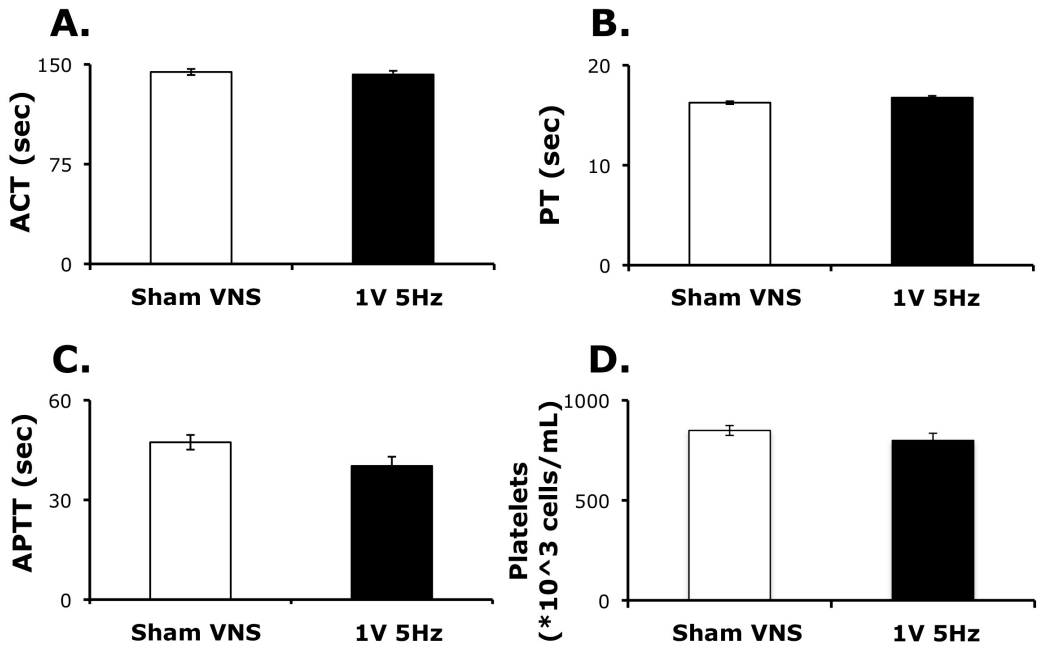
(B) BALB/c mice received electrical vagus nerve stimulation (1 V, 5 Hz, 2 ms pulse width) for 30, 60, 300, or 1200 sec (black bars), or sham electrical stimulation (n=10/group) (white bar); bleeding times following partial tail resection were determined. Bleeding time was recorded in seconds, and is presented as mean +/- SEM. Student's t-test was used to compare the vagus nerve-stimulated groups to the sham-stimulated group (*: $p<0.05$).


A.
Bleeding Time (sec)
200
160
120
80
40
0
*
Control
1V, 5Hz
5V, 30 Hz
B.
Bleeding Time (sec)
250
200
150
100
50
0
*
*
*
*
0
0.5
1.0
5.0
20
VNS Time (min)

One potential mechanism for the observed effects of vagus nerve stimulation on peripheral bleeding time is activation of the coagulation factor cascades, which are activated rapidly after platelet plug formation within a wound. However, no significant differences were found in coagulation times of circulating blood from animals that received vagus nerve stimulation or sham stimulation (Figure 13A-C). Moreover, no differences in the number of circulating platelets were observed following vagus nerve stimulation (Figure 13D).

**Figure 13: Electrical vagus nerve stimulation does not alter coagulation activity of circulating blood.**

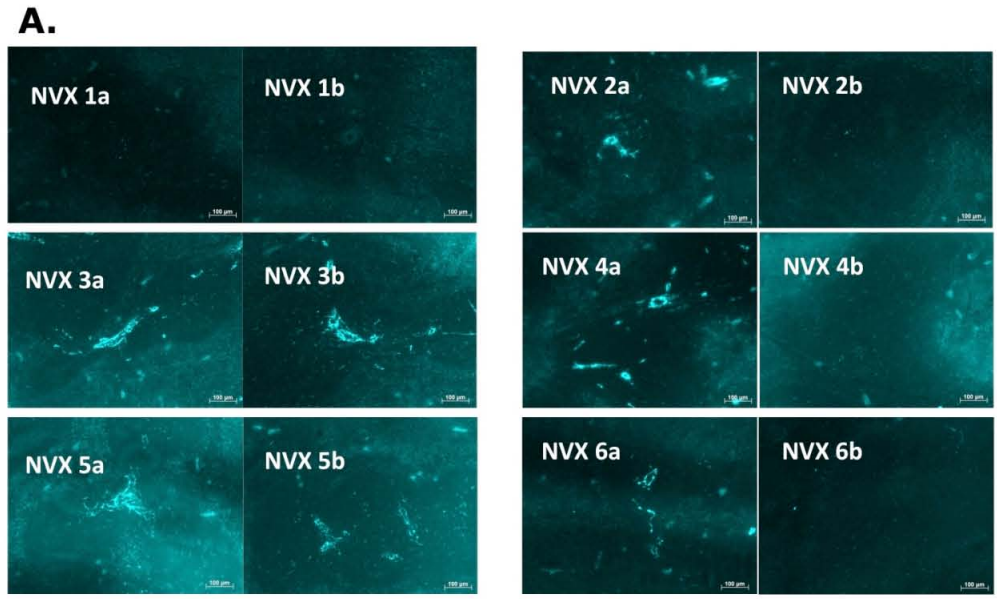
(A) BALB/c mice received 30 sec of electrical stimulation (1 V, 5 Hz, black bar; n=9-10/group) or sham stimulation (white bar; n=10). Blood was collected via cardiac puncture 5 min after completion of vagus nerve stimulation or sham stimulation, and (A) activated coagulation time (ACT), (B) prothrombin time (PT), and (C) activated partial thromboplastin time (APTT) were measured, and (D) platelets counted. A-C were recorded in seconds, (D) was recorded as thousands of platelets per mL, and each is presented as mean +/- SEM. Student's t-test was used to compare the vagus nerve stimulated group to the sham stimulated group (*: $p<0.05$).


A.
ACT (sec)
150
75
0
Sham VNS
1V 5Hz
B.
PT (sec)
20
10
0
Sham VNS
1V 5Hz
C.
APTT (sec)
60
30
0
Sham VNS
1V 5Hz
D.
Platelets
(*10^3 cells/mL)
1000
500
0
Sham VNS
1V 5Hz

Our previous studies have demonstrated that the spleen is the primary target organ of the cholinergic anti-inflammatory pathway, and that catecholaminergic nerve fibers in the spleen are co-located with TNF-producing macrophages (82, 94). To determine whether electrical vagus nerve stimulation modulates hemostasis through the spleen, the splenic branches of the vagus nerve were transected along the splenic artery and the splenic vein. Seven days after surgery, animals were subjected to electrical vagus nerve stimulation. Monoaminergic nerve fibers were still evident in many of the spleen sections, indicating the low frequency of complete splenic neurectomy using this technique in this species (Figure 14). In other mice not subjected to neurectomy, vagus nerve stimulation induced the expression of four genes in the spleen, including a cell surface marker on platelets (CD72) and thrombospondin 4 (Table 4).

**Figure 14: Efficacy of splenic neurectomies is highly variable.**

BALB/c mice were subjected to splenic neurectomy (NVX) (A; n=6) or sham neurectomy (B; n=4); 7 days later, animals were subjected to electrical vagus nerve stimulation. One hour after vagus nerve stimulation, spleens were harvested, stained for monoaminergic nerve fibers, and examined under a fluorescent microscope. Two representative sections from each neurectomized mouse are shown (labeled as “a” and “b”). No catecholaminergic nerves were evident in NVX1, while sections from mouse spleens NVX3, NVX4, and NVX5 stained positive for catecholaminergic nerves throughout.


A.
NVX 1a
NVX 1b
NVX 2a
NVX 2b
NVX 3a
NVX 3b
NVX 4a
NVX 4b
NVX 5a
NVX 5b
NVX 6a
NVX 6b

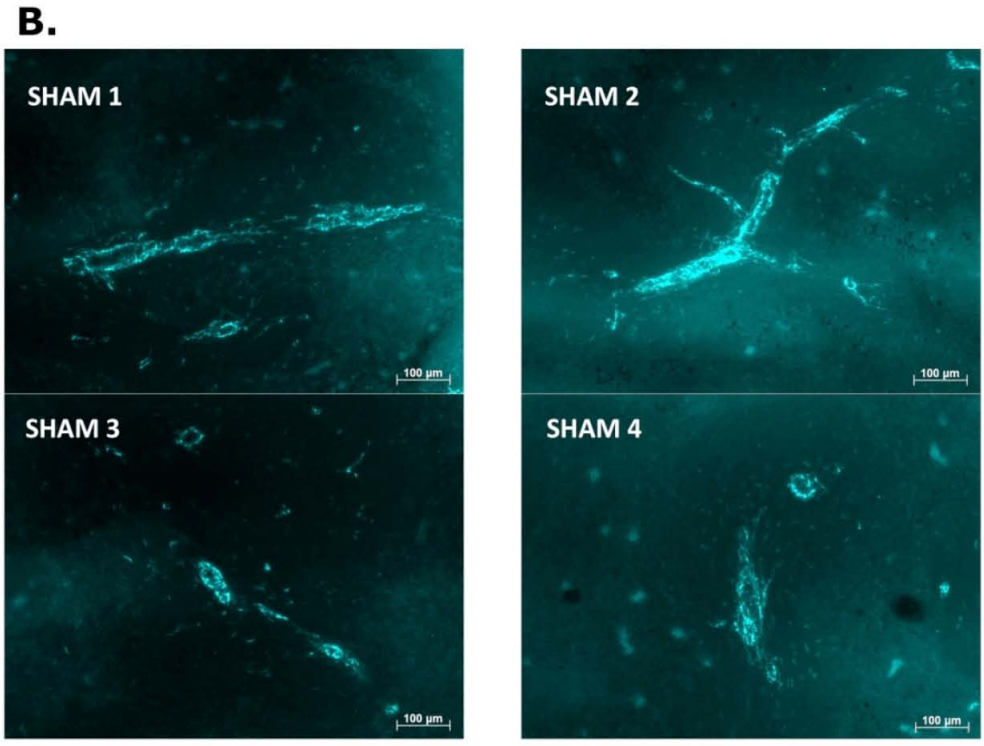

B.
SHAM 1
SHAM 2
SHAM 3
SHAM 4
100 µm

**Table 4: Vagus nerve stimulation-induced changes in splenic gene expression.**

| Symbol | Protein | Function | Fold Effect |
|---|---|---|---|
| Cd72 | CD72 | platlet membrane protein; B cell proliferation/differentiation | 7.44 |
| Thbs4 | thrombospondin 4 | protein family linked to thrombus formation and atherosclerosis | 2.02 |
| Otos | otospiralin | Inner ear protein | 2.56 |
| LOC503073 | unknown | RNA with sequence similarity to Igh-1a | 2.03 |

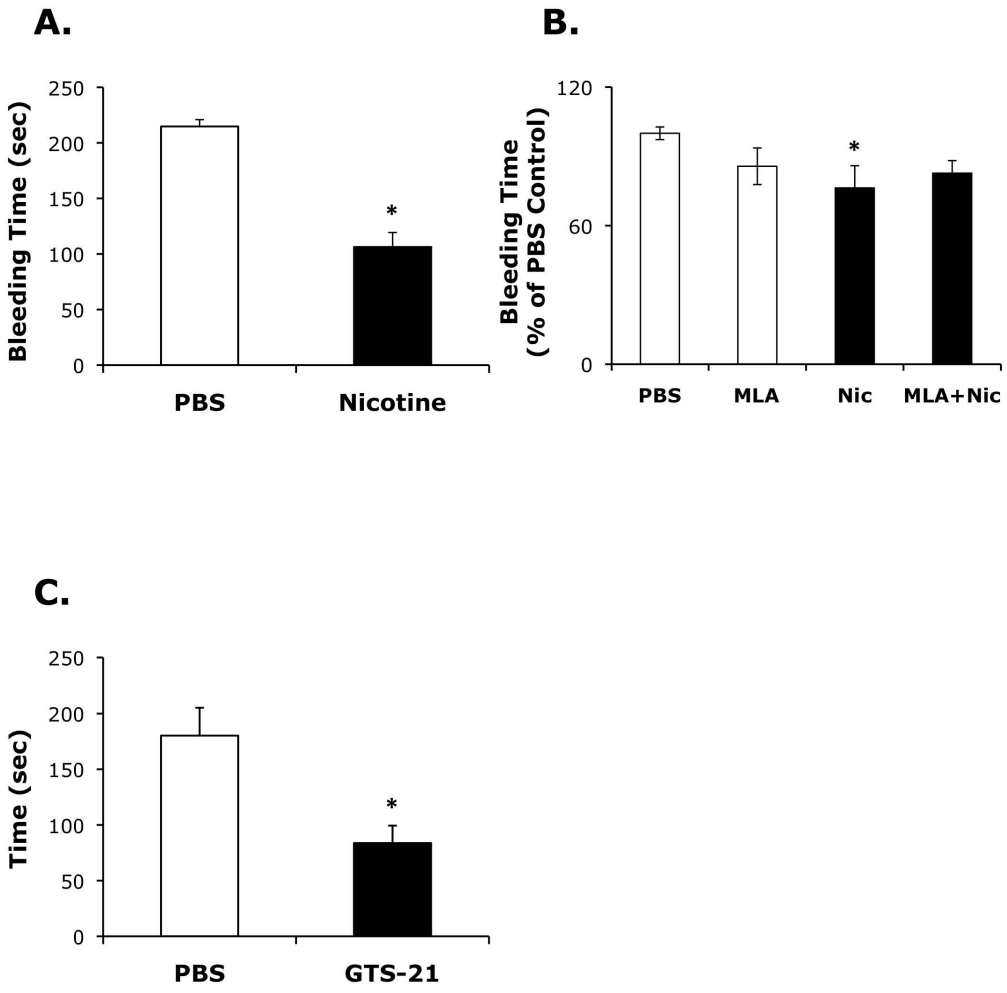
All studies above were conducted in anesthetized mice, raising the possibility that vagus nerve stimulation activated a neural pathway that had become blunted by anesthesia. To rule out this possibility, we tested nicotine in conscious animals. Pre-treatment with nicotine significantly attenuated bleeding time (vehicle = 215 +/- 6.1 sec vs. nicotine = 106 +/- 13 sec; $p<0.001$; Figure 15A), suggesting that cholinergic signaling can regulate hemostasis in the absence of anesthesia. We next used methyllycaconitine (MLA), a selective antagonist of the α7 subunit of the acetylcholine receptor, to determine whether nicotine regulated hemostasis through the α7 subunit of the acetylcholine receptor (262). Nicotine was incapable of reducing bleeding when animals were pre-treated with MLA (Figure 15B), suggesting that the effects of nicotine on bleeding time are dependent on the α7 subunit of the acetylcholine receptor. The ability of pharmacological activation of nicotinic cholinergic signaling to regulate hemostasis was further confirmed using GTS-21, an α7-selective cholinergic agonist (263). GTS-21 significantly attenuated bleeding time (Figure 14C).

**Figure 15: Cholinergic signaling recapitulates the hemostatic activity of electrical vagus nerve stimulation.**

(A) BALB/c mice (n=5/group) were pre-treated with nicotine (0.3 mg/kg, IP; black bar) or phosphate buffered saline (PBS, IP; white bar). One h later, conscious mice were placed in a restraint tube and acclimated for 5 min; then, bleeding times following partial tail resection were determined. Bleeding time was recorded in seconds, and is presented as mean +/- SEM. Student's t-test was used to compare the nicotine-treated group to the PBS-treated group (*: $p<0.05$).

(B) Anesthetized BALB/c mice (n=3-5/group) were pre-treated with phosphate buffered saline (PBS, first white column), methyllycaconitine (MLA; 4 mg/kg, i.p.; second white column), nicotine (0.3 mg/kg, i.p.; first black column), or nicotine (0.3 mg/kg, i.p.) plus methyllycaconitine (MLA; 4 mg/kg, i.p.; second black bar). Animals first received MLA or vehicle (PBS), and 15 min later were injected with nicotine or vehicle (PBS); 1 h after administering nicotine, animals were anesthetized, and subjected to partial tail resection. Bleeding time was recorded in seconds, and is presented as percent of PBS-treated group +/- SEM. Student's t-test was used to compare each experimental group to the PBS-treated group (*: $p<0.05$).

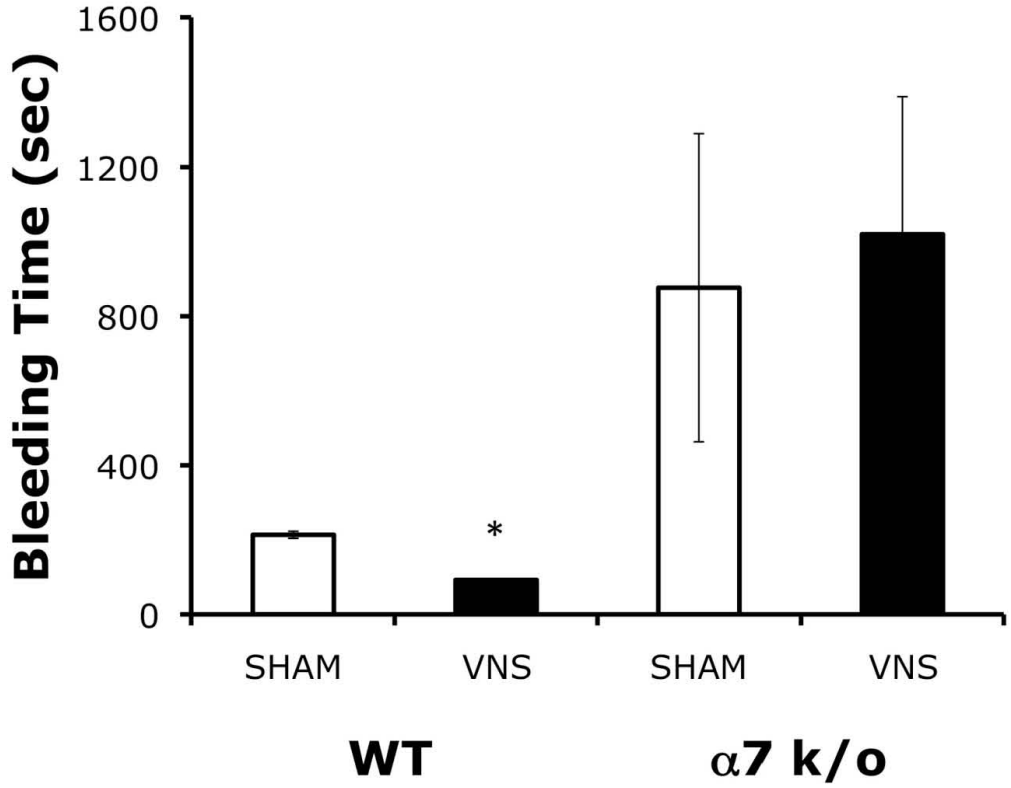
(C) Anesthetized BALB/c mice (n=3-5/group) were pre-treated with the cholinergic agonist GTS-21 (4 mg/kg, i.p); or vehicle (PBS); 1 h after administering GTS-21, animals were anesthetized, tails amputated, and bleeding time measured with a stopwatch. Bleeding time was recorded in seconds, and is presented as mean +/- SEM. Student's t-test was used to compare the GTS-21-treated group to the PBS-treated group (*: $p<0.05$).


A.
Bleeding Time (sec)
250
200
150
100
50
0
*
PBS
Nicotine
B.
Bleeding Time
(% of PBS Control)
120
60
0
*
PBS
MLA
Nic
MLA+Nic
C.
Time (sec)
250
200
150
100
50
0
*
PBS
GTS-21

The observation that the acetylcholine receptor-specific antagonist MLA could abrogate the hemostatic effects of nicotinic cholinergic agonists suggests that the α7 subunit of the acetylcholine receptor contributes to the neural regulation of hemorrhage. In order to better understand the potential role of α7 in this pathway, we applied vagus nerve stimulation to α7-deficient mice subjected to our tail resection model of peripheral hemorrhage. Mice deficient in the α7 subunit had significantly longer bleeding times than wild-type mice, and vagus nerve stimulation failed to attenuate peripheral bleeding time in these mice (sham stimulation = 1020+/-369 sec vs. vagus stimulation = 827+/-413 sec; $p<0.001$) (Figure 16).

**Figure 16: The α7 subunit of the nicotinic acetylcholine receptor subunit is an essential regulator of hemostasis.**

Wild type mice (WT; n=5/group) or α7nAChR-deficient mice (α7 k/o; n=4/group) received 30 sec of electrical stimulation (1 V, 5 Hz, 2 ms pulse width; black columns) or sham stimulation (white columns), and were then subjected to partial tail resection. Bleeding time was recorded in seconds, and is presented as mean +/- SEM. Student's t-test was used to compare the vagus nerve-stimulated groups to the sham-stimulated groups (*: $p<0.05$).


Bleeding Time (sec)
1600
1200
800
400
0
*
SHAM
VNS
SHAM
VNS
WT
α7 k/o

# Discussion

## Identification and characterization of a cholinergic anti-inflammatory pathway in pigs

The cholinergic anti-inflammatory pathway regulates pro-inflammatory cytokine release through a pathway dependent on the vagus nerve. We have demonstrated previously that this pathway can be activated by electrical and mechanical stimulation of the vagus nerve, and that these approaches inhibit the release of pro-inflammatory cytokines, including TNF and HMGB1, and improve survival in a murine model of severe sepsis (62). In mice, the cholinergic anti-inflammatory pathway requires a very low activation threshold, suggesting that the A fibers of the vagus nerve carry the anti-inflammatory activity of this pathway (81). This provides an opportunity to specifically activate the cholinergic anti-inflammatory pathway without affecting other systems regulated by the vagus nerve, such as the heart rate and blood pressure. To define stimulation parameters that affect heart rate in a large animal model, electrical stimulation was applied the vagus nerves of anesthetized pigs: 10 mA of electricity was sufficient to slow and even temporarily stop the heart; in contrast, 3.5 mA had only a slight, statistically insignificant effect on heart rate and mean arterial blood pressure. These results identified electrical vagus nerve stimulation parameters that could be used with minimal cardiac effect.

To determine whether electrical stimulation parameters at or below the cardiac threshold could activate the cholinergic anti-inflammatory pathway, anesthetized pigs received electrical vagus nerve stimulation and were then subjected to endotoxemia. The stimulation parameters used in these experiments significantly reduced circulating levels of TNF and prevented loss of blood pressure, two primary characteristics of endotoxemic shock. In pigs without endotoxin infusion, these same parameters did not significantly affect heart rate or blood pressure. In addition, animals received fluid resuscitation titrated in order to maintain blood pressure >50 mmHg and <70 mmHg. Animals that received electrical vagus nerve stimulation required significantly less fluid resuscitation than animals that received sham stimulation procedures, indicating that vagus nerve stimulation prevented the development of endotoxemic shock. Taken together, these results suggest that pigs possess an intact cholinergic anti-inflammatory pathway that is carried on vagus nerve fibers distinct from those that innervate the heart.



Endnotes can be found in the Literature Cited, beginning on page 104.

Coagulopathy is a well-known complication of sepsis and septic shock, and occurs in murine and porcine models of endotoxemia (250, 264). Endotoxin can cause expression of tissue factor by circulating monocytes and other cells, thereby triggering disseminated intravascular coagulation (265, 266). Antibodies to inflammatory cytokines can prevent disseminated intravascular coagulation and improve survival in animal models of sepsis, indicating that an overwhelming inflammatory response to infection and bacterial products activates systemic coagulation (267, 268). In our studies with pigs, electrical vagus nerve stimulation, using the parameters described above, prevented an endotoxin-induced decrease in circulating platelets, and also prevented the endotoxin-induced increase in circulating thrombin-anti-thrombin III (TAT) complex levels, both of which are accepted markers of coagulopathy during sepsis and endotoxemia. These observations are consistent with the TNF-suppressing effects of electrical vagus nerve stimulation in porcine endotoxemia, and further support the conclusion that activation of the cholinergic anti-inflammatory pathway has therapeutic benefit in a large animal species.

The lungs of pigs have abundant pulmonary intravascular macrophages, which release large quantities of thromboxane $A_2$ and other vasoconstrictors in response to endotoxin (269, 270). As a result, pigs demonstrate a profound and rapid increase in pulmonary arterial pressure and reduced cardiac output during endotoxemia (271). In our model of porcine systemic inflammation, endotoxin was infused in increasing amounts over 60 min; although this approach avoids dramatic increases in pulmonary artery pressure and cardiac failure, increased pulmonary artery pressure and reduced cardiac output was still observed. Electrical vagus nerve stimulation had no significant effect on pulmonary artery pressure or cardiac output during porcine endotoxemia. Earlier studies of the cholinergic anti-inflammatory pathway in mice have failed to reveal an effect of vagus nerve stimulation on cytokine levels in the lungs of rats (67). Since the B and C fibers of the vagus nerve are considered to be primary routes of innervation to the heart and lungs, and since the cholinergic anti-inflammatory pathway is principally carried by A fibers (81), it is reasonable to consider that the vagus nerve does not supply anti-inflammatory fibers to the lung.

## Vagus nerve stimulation attenuates bleeding time in pigs

The identification of an active cholinergic anti-inflammatory pathway in pigs provided the opportunity to study the effects of vagus nerve stimulation on hemostasis. Our studies indicate that electrical vagus nerve stimulation attenuated peripheral hemorrhage and reduced shed blood volume in pigs. The effects of vagus nerve stimulation on bleeding time were independent of effects on heart rate or blood pressure, suggesting that the vagus nerve may directly or indirectly modulate the coagulation activity of blood.

Initial experiments were designed to develop a porcine model of peripheral hemorrhage, which would provide a standardized wound with reproducible bleeding times and provide the opportunity to collect shed blood for analysis. We also sought to induce injury in a bilateral fashion, so that analyses could be performed on one wound site; the vagus nerve could then be stimulated; and a second wound inflicted and analyzed after vagus nerve stimulation. A model of forelimb cuticular injury was first tested, as this tissue is highly vascularized and innervated (272). This model proved to be technically challenging, because the tissue in this area of the forelimb is particularly tough and difficult to cut, even with a new surgical scalpel. As a result, bleeding times were highly inconsistent, potentially masking any hemostatic effect of vagus nerve stimulation. Other cutaneous injury models were also attempted, including punch biopsies and template incisions made with scalpels or dermatomes. These models failed to elicit significant bleeding times, and shed blood volumes did not exceed approximately 100 µL. Pilot experiments for non-compressible models of hemorrhage, including spleen and liver injury, were also performed. The use of an X-shaped aluminum rod created standardized, reproducible organ defects. Bleeding time in this model was protracted, and collection of blood for analysis proved challenging. Although set aside for the studies described here, this model proved useful for preclinical testing of gelatin thrombin granules (FloSeal) (250).

We next tested the ears as a potential site for bilateral soft tissue injury, as these tissues are also highly vascularized (272). Since ears are important thermoregulatory organs, tissue-surface temperature was carefully regulated with a heat lamp, and monitored with the use of a non-contact infrared thermometer. The wounds were easily standardized, because a defined section of tissue could be completely amputated. Initial experiments indicated that the ears provided reproducible bleeding times, and provided sufficient volumes of blood for ex vivo analysis. Using this partial ear resection model of peripheral hemorrhage, we demonstrated that bleeding times, shed blood volume, and bleeding rates were

significantly reduced following vagus nerve stimulation. Of note, the vagus nerve does provide both sensory and motor fibers to the human ear; whether these fibers exist in pigs is unknown. In addition, electrical stimulation was applied to the cervical region of the vagus nerve, and no mechanisms were in place to prevent activation of afferent signals. This raises the possibility that electrical vagus nerve stimulation induced afferent signals, which activated a central signaling arch that, through the brain, resulted in efferent activation of vagus or other motor neurons.

Prothrombin is produced in hepatocytes and other cells, and constitutively released into the systemic circulation as a proto-enzyme. At the site of vascular injury, activated factor X cleaves prothrombin on the surface of platelets to release activated thrombin, which accelerates the generation of fibrin and clot deposition (273). Anti-thrombin is a constitutively-produced thrombin inhibitor, which maintains an anti-coagulant state by binding activated thrombin as it diffuses from the site of vascular injury (274, 275). Measurement of TAT complex levels is an indirect but sensitive and widely accepted measure of thrombin generation. Here, vagus nerve stimulation failed to significantly alter TAT complex levels in the circulation at any time point in the study, but vagus nerve stimulation significantly increased the level of TAT complex found in blood shed from the wound site, as compared with blood shed from the contralateral wound prior to vagus nerve stimulation. No significant increase in TAT complex levels was observed in shed blood following a sham stimulation procedure. The change in bleeding time induced by vagus nerve stimulation correlated with changes in TAT complex levels in shed blood, suggesting that increased thrombin formation contributed to the observed reduction in bleeding time. The observation that vagus nerve stimulation increased TAT complex formation specifically in shed blood, but not in the systemic circulation, suggests that efferent vagus nerve activity directly or indirectly modulates thrombin activity at the site of injury. An alternative explanation for the observed results is that, following vagus nerve stimulation, blood flowed more slowly across the wound site, and became enriched for TAT complexes. Additional experiments are required to test this possibility.

Platelet aggregation and rotational thromboelastography (RoTEG) assays were employed to assess the effect of vagus nerve stimulation on blood coagulation activity. In the RoTEG data presented here, “r” time, or the reaction time to initiate clot formation; “k” time, or the coagulation time; and maximum amplitude (MA), or the maximum viscosity of the clot, were measured in both the general circulation and in shed blood, before and after sham or electrical vagus nerve stimulation. Reduced platelet

aggregation in the circulation was observed in both groups, but reached statistical significance ($p<0.05$) only in the sham group; k time and maximum amplitude in circulating blood were not significantly changed by vagus nerve stimulation; r time was significantly shortened following vagus nerve stimulation. RoTEG analyses of shed blood revealed no statistically significant changes in clotting activity following either sham or vagus nerve stimulation. While vagus nerve stimulation modulates r time in circulating blood, the mechanism of this effect has yet to be defined.

Previous studies have shown that electrical or pharmacological stimulation of the cholinergic anti-inflammatory pathway significantly protects rodents against the pathological sequelae of hypovolemic shock and improves survival (276). The model of peripheral hemorrhage described here does not induce hemorrhagic shock; less than 1% of total blood volume was shed. The peripheral injuries induced in our model are potentially controllable through more conventional methods, such as direct compression or application of a tourniquet. Whether vagus nerve stimulation can be used for non-compressible hemorrhage, such as internal (organ) hemorrhage, has yet to be determined.

Extrapolation of these findings to clinical trauma is further complicated by thermoregulation, fluid resuscitation, and anesthesia. In our studies, the temperature of the skin at the wound site was regulated to minimize the potential effect of thermoregulatory responses on vasodilatory status, bleeding time and volume of blood shed. In addition, these studies were conducted in the presence of general anesthesia, which has the potential to alter neurologic responses to injury. The design of these experiments could not identify any potential influence of anesthesia on the hemostatic effects of electrical vagus nerve stimulation. Nonetheless, these studies indicate that electrical stimulation of the vagus nerve decreases bleeding time and the volume of shed blood in porcine soft tissue injury.

## Vagus nerve stimulation attenuates bleeding time in mice

Efferent vagus nerve signals of the cholinergic anti-inflammatory pathway converge on the α7 subunit of the acetylcholine receptor, which is required to maintain immunological homeostasis (65). The observation that electrical vagus nerve stimulation regulates bleeding in porcine soft tissue injury raises the possibility that neural regulation of hemostasis similarly requires α7. In order to test this possibility, we first developed a murine model of soft tissue injury, based on partial tail resection, and tested the effects of electrical vagus nerve stimulation. In these studies, vagus nerve stimulation significantly reduced bleeding time as compared with animals subjected to a sham stimulation procedure, in which the vagus nerve was surgically exposed but not stimulated. The effects of vagus nerve stimulation on bleeding time are independent of effects on heart rate or blood pressure, suggesting that the vagus nerve may directly or indirectly modulate the coagulation activity of blood.

Initial studies of the hemostatic effects of vagus nerve stimulation used 20 minutes of stimulation, which significantly reduced bleeding time by 37% as compared with sham-stimulated animals ($p<0.001$). Time course analyses revealed that as little as 30 seconds of vagus nerve stimulation significantly reduced bleeding time, equally as effectively as longer durations of stimulation. In addition, two different electrical vagus nerve stimulation parameters (1 V and 5 V) were tested in this study. High levels of stimulation (5 V) decreased heart rate and microvascular flow in the periphery, as measured by laser Doppler studies, but had no effect on bleeding time. In contrast, low levels of stimulation (1V) had no measurable effect on heart rate and microvascular flow, but significantly reduced bleeding time. Together, these results indicate that vagus nerve stimulation rapidly attenuates bleeding responses to injury, independently of effects on heart rate and blood flow. These observations are consistent with our previous observations that electrical vagus nerve stimulation attenuates bleeding in pigs, and that reductions in bleeding time significantly inversely correlate with levels of thrombin-antithrombin (TAT) complex in blood shed from the wound.

To determine the effects of electrical vagus nerve stimulation on systemic coagulation activity, circulating blood collected via cardiac puncture 5 minutes after vagus nerve stimulation was assessed for coagulation activity, and platelets were counted. Neither the low (1 V) nor the high (5 V) stimulation parameters affected circulating platelet counts, or systemic coagulation measurements (activated clotting time, prothrombin time, or activated partial thromboplastin time). To rule out the possibility that cholinergic stimulation was activating endogenous

pathways blunted by the presence of anesthesia, we developed a conscious model of peripheral hemorrhage based on the partial tail resection technique. Nicotine significantly attenuated bleeding responses to injury in conscious mice. Together, these results indicate that the effects of vagus nerve stimulation on hemorrhage are independent of hemostatic effects in the general circulation.

Our studies of the cholinergic anti-inflammatory pathway have revealed that the spleen is an important source of pro-inflammatory cytokines during murine endotoxemia and sepsis, and that cholinergic signaling in vagus nerve converges on catecholaminergic signaling in the spleen (82, 94). We attempted similar studies to define the functional neuroanatomy of the cholinergic regulation of hemostasis. Because our primary endpoint (bleeding time) was not consistent in rats, we attempted splenic neurectomies in mice. Our observations indicate that splenic nerve fibers encircle both the splenic artery and splenic vein, making complete isolation and transection technically demanding; complete denervation of the spleen was achieved in less than 25% of all surgeries.

As an alternative approach to defining the functional neuroanatomy of autonomic regulation of hemostasis, samples of spleen were harvested after electrical or sham vagus nerve stimulation, and changes in gene expression analyzed on the Illumina array platform (258). Only four genes demonstrated a greater than four-fold increase in expression following electrical vagus nerve stimulation, two of which have potential roles in hemostasis. CD72 is a type II integral membrane protein that is expressed on the surface of platelets, and believed to enhance thrombin formation (277). The second gene product is the transcript for thrombospondin 4, a member of a family of proteins that have assorted roles in thrombus formation (278). Thrombospondin 4 regulates endothelial cell adhesion and proliferation, and may thus play a role in atherosclerosis; human genetic analyses have linked polymorphisms in thrombospondin 4 with increased rates of myocardial infarction (279, 280, 281). Our studies were unable to determine the cell types in which these genes are up-regulated following electrical vagus nerve stimulation, because we studied only total spleen extracts. An important consideration in the interpretation of these findings, however, is the kinetic response of this observation: harvested spleens were analyzed one hour after electrical vagus nerve stimulation, whereas bleeding was induced within five minutes of electrical vagus nerve stimulation. Further studies are required to more fully define the potential role of these gene products in autonomic regulation of hemostasis.

We have previously determined that pharmacological cholinergic signaling, activated by the cholinergic agonists nicotine, GTS-21, and choline, has significant anti-inflammatory activity, and attenuates pro-inflammatory cytokine release and improves survival in murine models of endotoxemia and sepsis (62). To determine whether cholinergic signaling was capable of recapitulating the hemostatic effects of vagus nerve stimulation, nicotine was administered to mice prior to partial tail resection under anesthesia. Doses of nicotine that are therapeutic in models of systemic inflammation and sepsis (66) significantly reduced bleeding time in this model of peripheral hemorrhage. MLA, a selective antagonist of the α7 subunit of the cholinergic receptor (282), significantly inhibited the protective effects of nicotine, demonstrating the receptor specificity of nicotine in the regulation of hemostasis. The knowledge that nicotinic cholinergic receptors regulate coagulation suggests that pharmacological approaches may be developed to provide a novel means of hemorrhage control in soft tissue injuries. Whether vagus nerve stimulation or cholinergic agonists can be used for non-compressible hemorrhage, such as internal (organ) hemorrhage, has yet to be determined.

The observation that cholinergic agonists can regulate hemostasis through the α7 subunit of the acetylcholine receptor suggested that electrical vagus nerve stimulation may also converge on this receptor, as we have demonstrated previously for the cholinergic anti-inflammatory pathway (65). To confirm this hypothesis, mice genetically deficient in the α7 subunit received electrical or sham vagus nerve stimulation, and were then subjected to partial tail resection. α7 knock-out mice failed to respond to vagus nerve stimulation, and had bleeding times that were not significantly different than animals that received sham stimulation. Moreover, α7 knock-out mice had significantly longer bleeding times than wild-type mice, suggesting that this acetylcholine receptor subunit is an essential component of an endogenous neural pathway that regulates hemostasis. These data indicate that the molecular events following vagus nerve stimulation converge on the α7 subunit of the nicotinic cholinergic receptor to modulate coagulation activity during soft tissue injury.

Implanted vagus nerve stimulators are used in humans for the treatment of epilepsy and depression that are refractory to other means of treatment (283, 284), an approach that would be impractical for an acute injury. To our knowledge, coagulation responses have not been studied in patients with implanted vagus nerve stimulators. We have recently shown that non-invasive, transcutaneous stimulation of the vagus nerve attenuates cytokine release and improves survival in a murine model of peritonitis/sepsis (81). Application of this approach to acute hemorrhage

and trauma can now be explored for possible adaptation for pre-hospital care of military or civilian casualties, because carotid massage is a clinically approved technique for the termination of cardiac tachyarrhythmias (285). Elucidation of the mechanism of action of the hemostatic activity of vagus nerve stimulation may reveal novel therapeutic approaches for a variety of trauma- and hemorrhage-related applications.

## The neural tourniquet: a newly identified endogenous mechanism that regulates hemostasis

Although the interactions between the sympathetic autonomic nervous system and coagulation have been studied for decades, less is known about the role of the parasympathetic system in regulating hemostasis (218, 230, 286, 287, 288, 289). The studies presented here indicate that the α7 subunit of the acetylcholine receptor is an essential regulator in a cholinergic pathway that modulates hemostasis, and bring to light the possibility that both branches of the autonomic nervous system function together as a "neural tourniquet" to regulate hemostasis. Appropriate regulation of this pathway may have important clinical significance, because amplified parasympathetic outflow coordinates restoration, recovery, and healing following injury (290). However, balanced autonomic modulation of the circulation is important for recovery, because high parasympathetic and low sympathetic tone is associated with intensive care unit and trauma mortality (291, 292).

It is now plausible to consider that the neural tourniquet is the motor arm of a reflex mechanism that regulates hemostasis (232). Most autonomic reflexes are comprised of parasympathetic and sympathetic systems functioning in opposition, thus allowing rapid and precise control over physiologic activity. While previous studies have demonstrated that electrical vagus nerve stimulation induces the release of both pro-and anti-coagulant activities (234, 235, 236, 237, 238), we have demonstrated that efferent vagus nerve signals, converging on the α7 subunit of the acetylcholine receptor, favor a pro-thrombotic state in the context of injury. The neurotransmitters epinephrine and norepinephrine can affect coagulation indices in vivo and in vitro, implicating a role for the sympathetic nervous system in this pathway (293). Additional studies will be required to identify the sensory, or afferent, arm of this reflex mechanism, and to understand more clearly the differing effects that sympathetic and parasympathetic neural pathways may have on hemostasis.

Several important questions about the function and anatomy of the neural tourniquet remain unanswered. For example, while the vagus nerve extends both motor and sensory fibers to some peripheral tissues, many other tissues do not receive vagus innervation. Since many tissue sources of coagulation factors, including liver, bone marrow, and endothelium, receive primarily sympathetic innervation, it is reasonable to consider that the vagus nerve makes functional contacts with the sympathetic system to influence hemostasis, as has been found for neural regulation of immunity (94, 294). Neural activity in these and/or other

organs could influence hemostasis at distal sites through diffusible mediators, which may include thrombospondin 4, or perhaps neurotransmitters or their metabolic products, such as choline (108). Although no effect on ex vivo platelet aggregation was observed in our studies, we did find that electrical vagus nerve stimulation increased expression of CD72, a plalelet and B cell membrane protein, in whole spleen extracts.

It is reasonable to consider that circulating platelets may be "educated" by the neural tourniquet in organs such as the liver, spleen or kidney, as we have observed for macrophages in studies of the cholinergic anti-inflammatory pathway (81). Platelets express receptors for the sympathetic neurotransmitters epinephrine and norepinephrine (217, 224, 225), indicating that they are capable of receiving catechholaminergic signaling in the spleen (94). Platelets also express mRNA for nicotinic cholinergic receptors, but whether the proteins are expressed is unclear (295, 296). Our studies of the cholinergic innervation of the immune system suggest that target cells of the cholinergic anti-inflammatory pathway receive neural signals via catecholaminergic signaling, and that the role of α7 in this pathway likely resides within a ganglionic synapse (82, 94). Further elucidation of the effect of neurotransmitter signaling on the coagulation cascade and platelet activity/gene expression will help define the molecular mechanism of this pathway.

The identification of the neural tourniquet provides the opportunity to develop new strategies to control hemorrhage. Our previous studies indicate that percutaneous stimulation of the vagus nerve is sufficient to activate the cholinergic anti-inflammatory pathway (81); if proven effective in activating the neural tourniquet, percutaneous vagus nerve stimulation may be an effective treatment for trauma-related hemorrhage. Continued studies of α7-selective cholinergic agonists may identify pharmacological hemostatic agents that may prove useful in the clinical management of trauma hemorrhage, and perhaps in hemophilia (297, 298, 299). It should be noted that the hemorrhage models employed in these studies were minor soft-tissue injuries, and were potentially controllable by direct pressure or traditional tourniquet to stop bleeding. The neural tourniquet will need to be studied in more robust hemorrhage models to determine its applicability to significant human trauma.

The knowledge that the vagus nerve can influence hemostasis expands the spectrum of functions of the autonomic nervous system, and provides opportunities to develop novel therapeutics. This discovery also raises the possibility that other, as yet unidentified, reflexive functions of

the autonomic nervous system modulate normal physiology. Recent evidence suggests that the autonomic nervous system regulates inflammation and hemostasis, and may play a role in cardiovascular disease, angiogenesis, body weight, physical performance, and neurocognition (300, 301, 302, 303, 304). As with heart rate, neural regulation of variability in organ function is an important aspect of health, and disruptions in variability can be both a cause and a symptom of developing disease.

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