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Background—We investigated whether electrical stimulation (STIM) of efferent vagus nerves may suppress nuclear factor (NF)-κB activation and the inflammatory cascade in hemorrhagic (Hem) shock.

Methods and Results—Rats were subjected to bilateral cervical vagotomy (VGX) or sham surgical procedures. Hem shock was induced by intermittent withdrawing of blood until mean arterial pressure stabilized within the range of 35 to 40 mm Hg. Application of constant voltage pulses to the caudal vagus ends (STIM; 5 V, 2 ms, 1 Hz for 12 minutes, 5 minutes after mean arterial pressure stabilization) increased survival time (VGX+Hem+Sham STIM=38±3 minutes; VGX+Hem+STIM >180 minutes), reverted the marked hypotension (VGX+Hem+Sham STIM=33±3 mm Hg; VGX+Hem+STIM=66±5 mm Hg), inhibited IκBα liver loss, and blunted the augmented NF-κB activity, decreased hepatic tumor necrosis factor (TNF)-α mRNA (VGX+Hem+Sham STIM=1.42±0.5 amount of TNF-α m-RNA; VGX+Hem+STIM=0.51±0.2 amount of TNF-α mRNA), and reduced plasma TNF-α (VGX+Hem+Sham STIM=190±24 pg/mL; VGX+Hem+STIM=87±15 pg/mL). Chlorisondamine, a nicotinic receptor antagonist, abated the effects of vagal stimulation.

Conclusions—Our results show a parasympathetic inhibition of NF-κB by which the brain opposes NF-κB activation in the liver and modulates the inflammatory response during acute hypovolemic hemorrhagic shock. (***Circulation***. 2003; **107:1189-1194.**)

Key Words: vagus nerve ■ hemorrhage ■ shock ■ acetylcholine ■ inflammation

Hemorrhagic shock initiates an inflammatory response characterized by the upregulation of cytokine expression[1] and accumulation of neutrophils in a variety of tissues.[2] These changes are prominent in the lungs and liver and contribute to end-organ damage and resultant dysfunction after shock.

Nuclear factor κB (NF-κB) is an ubiquitous rapid response transcription factor involved in inflammatory reactions and exerts its actions by expressing several cytokines, chemokines, and cell adhesion molecules (vascular cellular adhesion molecule-1 and intracellular adhesion molecule-1).[3] Several studies indicate that NF-κB–triggered inflammatory cascade becomes early activated during acute hemorrhage, even in absence of resuscitation procedures.[4–6] This inflammatory cascade causes a marked production of tumor necrosis factor-α

(TNF-α) that plays a pivotal role in the vascular failure and in the end-organ damage of acute hypovolemic shock.[5,6]

The central nervous system modulates systemic inflammatory responses to various stressors through humoral mechanisms.[7–9] Stimulation of afferent vagus nerve fibers by cytokines or endotoxin stimulates pituitary-adrenal antiinflammatory responses[10–12]; however, a role of efferent vagus nerve signaling also exists in modulating inflammation.

Efferent vagus nerve signaling reduces the inflammatory response in septic shock. Indeed, peripheral vagus nerve electrical stimulation during lethal endotoxemia blunted hepatic TNF-α synthesis, attenuated peak serum cytokine, and prevented shock development.[13] Furthermore, acetylcholine significantly reduces the release of several inflammatory cytokines in lipopolysaccharide-stimulated human macro-

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phage cultures, and this effect is abated by a nicotinic receptor antagonist.[13]

The mechanism by which the systemic release of TNF-α is controlled during acute hypovolemic hemorrhagic shock in the rat has not been fully elucidated. Liver activation of a NF-κB–mediated pathway causes an increased hepatic TNF-α production. In analogy to endotoxin shock,[13] the cholinergic antiinflammatory pathway might also directly modulate the systemic response in acute hypovolemic hemorrhagic shock, in turn reducing the inflammatory cascade, leading to inflammatory cytokine production and vascular derangement. We therefore studied whether direct stimulation of efferent vagus nerves may suppress NF-κB activation and the subsequent inflammatory cascade in acute hypovolemic hemorrhagic shock.

Methods

Acute Hypovolemic Hemorrhagic Shock Protocol
Male Wistar rats (260 to 280 g body weight) (Harlan, Milan, Italy) were used with food in pellets (0.01% content of α-tocopherol) and tap water available ad libitum. Housing conditions and experimental procedures were in strict accordance with European Community regulations (CEE Council 89/609; Italian D.L.: 22–1–92 No. 116).

Under general anesthesia (urethane, 1.25 g/kg intraperitoneally) and after heparinization (heparin sodium, 600 IU/kg), rats were instrumented with indwelling polyethylene catheters in the left common carotid artery to record arterial blood pressure and into the right iliac vein to inject drugs and to bleed.

The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure was displayed on a polygraph (Mortara-Rangoni). Arterial blood pressure is reported as mean arterial pressure (MAP).

Acute hypovolemic hemorrhagic shock[14–16] was induced by a graded withdrawal of blood (1.8 to 2 mL per 100 g body weight) until the MAP fell and stabilized at levels of 35 to 40 mm Hg. Sham-shocked rats underwent all surgical procedures experienced by the hemorrhage-shocked animals, but they were not bled.

Bilateral Cervical Vagotomy, Electrical Stimulation, and Survival Evaluation
Two minutes before the start of bleeding, rats were subjected to bilateral cervical vagotomy or sham surgical procedures. Direct stimulation of both caudal vagal trunks was carried out by means of bipolar platinum electrodes connected to a stimulator. Constant voltage stimuli (STIM; 5 V, 2 ms, 1 Hz) or sham STIM were applied to nerves for 12 minutes,[13] starting 5 minutes after MAP stabilized at a level of 35 to 40 mm Hg. The subcutaneous pretreatment with chlorisondamine diiodide (0.125 mg/kg in saline, 1 mL/kg; Tocris Cookson Ltd) was carried out 2 minutes before the start of bleeding. Survival rate and survival time were evaluated for 180 minutes after the bleeding was discontinued.

Isolation of Nuclear and Cytoplasmatic Proteins
Briefly, 70 mg of pulverized liver samples (obtained 20 minutes after bleeding or sham bleeding discontinuation and 2 to 3 minutes after the end of STIM or sham STIM) were homogenized. Nuclear and cytoplasmatic proteins were isolated as previously described.[6]

Electrophoretic Mobility Shift Assay
NF-κB binding activity was performed as previously reported.[6] The binding bands were quantified by scanning densitometry of a bio-image analysis system (Bio-Profil Celbio).

The results for each time point from each group were expressed as relative integrated intensity compared with the sham operated group liver measured in the same batch, because the integrated intensity of group samples from different electrophoretic mobility shift assay batches would be affected by the half-life of the isotope, exposure time, and background levels.

Western Blot Analysis of IκBα in Cytoplasm
IκBα expression was studied as previously reported.[6] The IκBα protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil). The results from each experimental group were expressed as relative integrated intensity compared with normal livers measured with the same batch.

RNA Isolation and Reverse Transcriptase–Polymerase Chain Reaction
Total cellular RNA was extracted[6] from liver section 20 minutes after bleeding or sham bleeding discontinuation (and 2 to 3 minutes after the end of STIM or sham STIM). The following oligonucleotide pairs were used (5′ oligo/3′oligo), each sequence as 5′ to 3′: TNF-α: CACGCTCTTCTGTCTTACTGA/GGACTCCGTGATGTCTA-AGT;GAPDH:ACCACCATGGAGAAGGTCGG/CTCAGTG-TAGCCCAGGATGGC. A portion of the PCR product was electrophoresed and transferred to a nylon membrane that was prehybridized with oligonucleotide probes, radiolabeled with [^{32}P] ATP by a T4 oligonucleotide kinase.

After an overnight hybridization at 55°C, filters underwent the autoradiography in a darkroom with a fixed camera. The captured image, sent to an image analysis software (Bio-Profil), was subjected to densitometric analysis.

Plasma TNF-α Levels
Blood (750 μL) was drawn 20 minutes after bleeding or sham bleeding discontinuance (and 2 to 3 minutes after the end of STIM or sham STIM). Plasma TNF-α concentrations were determined by an ELISA kit (Genzyme).[6]

Statistical Analysis
Data are expressed as mean$\pm$SD and were analyzed by ANOVA for multiple comparison of results; Duncan's multiple range test and post-hoc evaluation were used to compare group means. In all cases, $P<0.05$ was selected as criterion for statistical significance. For survival rate, statistical analysis was done with Fisher's exact probability test.

Results

Effects of Vagus Nerve Stimulation on Activation of Nuclear Factor-κB in the Liver
NF-$\kappa\beta$ binding activity was present at very low levels in sham-shocked animals (15$\pm$1.7 integrated intensity). NF-κB activity was increased in rats subjected to the hemorrhagic procedures (80$\pm$11 integrated intensity; t value=17.49; F value=308.39; $P<0.005$; Figure 1).

Bilateral cervical vagotomy slightly increased NF-κB binding (90$\pm$12 integrated intensity; Figure 1). Application of constant voltage pulses to the caudal vagus ends blunted NF-κB activation (30$\pm$4 integrated intensity; t value=15.92; F value=194.59; $P<0.005$; Figure 1). Chlorisondamine pretreatment, at a dose able to block only peripheral nicotinic receptors,[17] reverted the effects of the vagal stimulation (74$\pm$9 integrated intensity; t value=11.92; F value=208.77; $P<0.005$; Figure 1).

Effects of Vagus Nerve Stimulation on the Loss of IκBα Protein in the Liver Cytoplasm
IκBα levels were studied 20 minutes after the bleeding was discontinued. The inhibitory protein disappeared from the liver cytoplasm in rats subjected to the acute hypovolemic
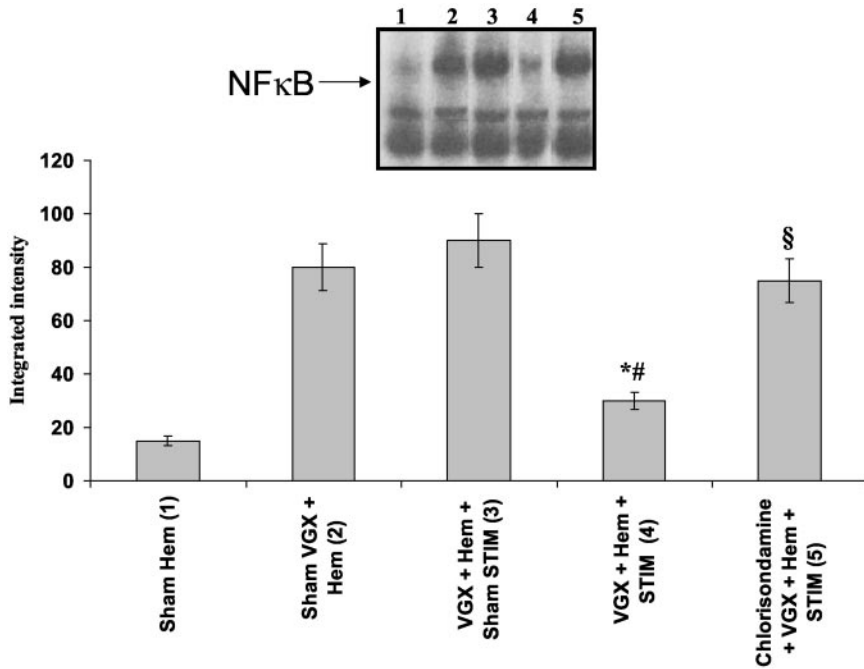
Figure 1. Electrophoretic mobility shift assay of NF-κB in rat liver. Rats underwent hemorrhagic shock (Hem) or sham shock (Sham Hem). Hem shock animals were either vagotomized (VGX+Hem) or sham vagotomized (Sham VGX+Hem). Hemorrhaged animals subjected to vagotomy underwent the application of constant voltage pulses to the caudal vagus ends (STIM) or a sham stimulation (Sham STIM). Chlorisondamine (0.125 mg/kg) was injected subcutaneously 2 minutes before the start of bleeding. Each point represents the mean±SD of 10 animals. *$P<0.005$ vs Sham VGX+Hem; #$P<0.005$ vs VGX+Hem+Sham STIM; §$P<0.005$ vs VGX+Hem+STIM.

hemorrhagic shock (3±0.9 integrated intensity), and bilateral cervical vagotomy did not modify IκBα loss (2±0.5 integrated intensity; Figure 2).

In contrast, vagus nerve stimulation blunted the consistent loss of IκBα protein from the liver cytoplasm (7±1.9 integrated intensity; t value=8.224; F value=64.77; $P<0.005$; Figure 2). Chlorisondamine administration reverted the effects of vagal stimulation (3±0.4 integrated intensity; t value=6.580; F value=42.44; $P<0.005$ Figure 2).

Effects of Vagus Nerve Stimulation on TNF-α mRNA Expression
Hepatic TNF-α mRNA expression was augmented in hemorrhaged animals with (1.2±0.4 relative amount of hepatic TNF-α mRNA) or without bilateral cervical vagotomy (1.4±0.5 relative amount of hepatic TNF-α mRNA; Figure 3).

Vagus nerve stimulation blunted hepatic TNF-α mRNA expression (0.51±0.2 relative amount of hepatic TNF-α mRNA; t value=6.186; F value=28.56; $P<0.005$). Hemorrhage-shocked rats pretreated with chlorisondamine did not show significant reduction in the increased mRNA expression (1.18±0.3 relative amount of hepatic TNF-α mRNA; t value=5.789; F value=34.53; $P<0.005$ Figure 3).

Effects of Vagus Nerve Stimulation on Plasma TNF-α Levels
Sham-shocked rats had very low levels of TNF-α (15±7 pg/mL; Figure 4). In hemorrhaged animals, the plasma levels of the inflammatory cytokine markedly increased 20 minutes after the bleeding was discontinued (180±19 pg/mL; t value=20.554; F value=664.02; $P<0.005$; Figure 4). Bilat-
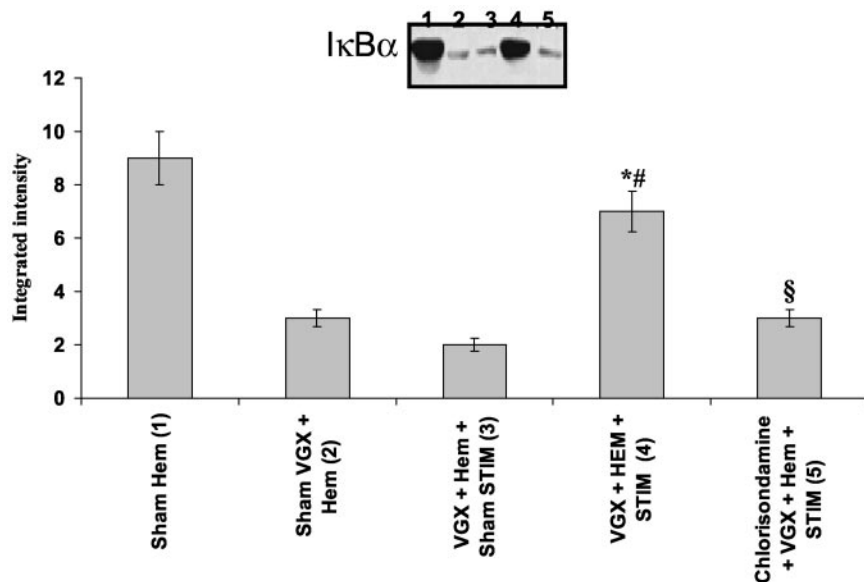
Figure 2. IκBα protein expression in rat liver. Rats underwent hemorrhagic shock (Hem) or sham shock (Sham Hem). Hem shock animals were either vagotomized (VGX+Hem) or sham vagotomized (Sham VGX+Hem). Hemorrhaged animals subjected to vagotomy underwent the application of constant voltage pulses to the caudal vagus ends (STIM). Chlorisondamine (0.125 mg/kg) was injected subcutaneously 2 minutes before the start of bleeding. Each point represents the mean±SD of 10 animals. *$P<0.005$ vs Sham VGX+Hem; #$P<0.005$ vs VGX+Hem+Sham STIM; §$P<0.005$ vs VGX+Hem+STIM.
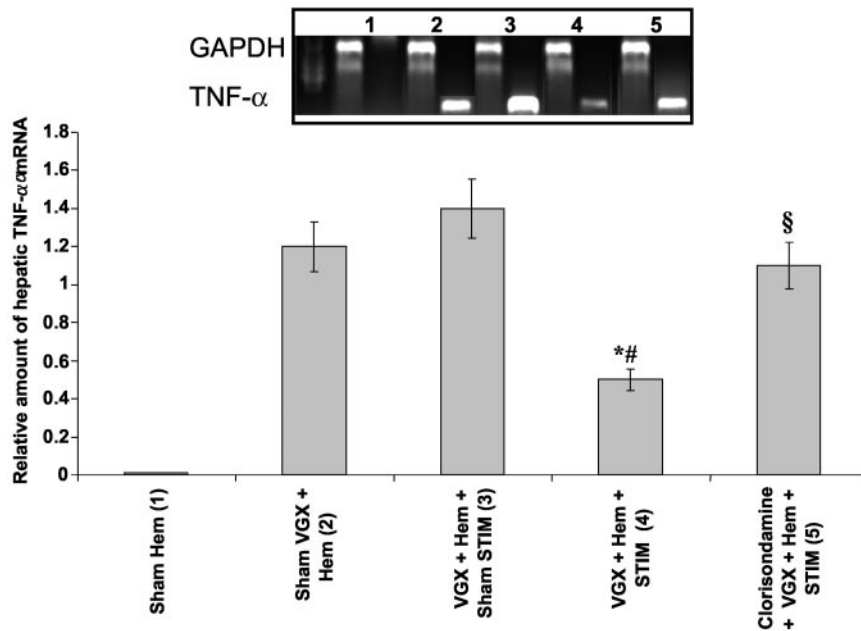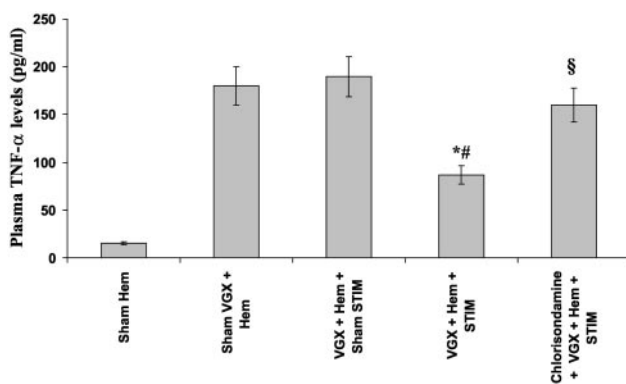
Figure 3. TNF-α mRNA in rat liver. Rats underwent hemorrhagic shock (Hem) or sham shock (Sham Hem). Hem shock animals were either vagotomized (VGX+Hem) or sham vagotomized (Sham VGX+Hem). Hemorrhaged animals subjected to vagotomy underwent the application of constant voltage pulses to the caudal vagus ends (STIM) or a sham stimulation (Sham STIM). Chlorisondamine (0.125 mg/kg) was injected subcutaneously 2 minutes before the start of bleeding. Each point represents the mean±SD of 10 animals. *$P<0.005$ vs Sham VGX+Hem; #$P<0.005$ vs VGX+Hem+Sham STIM; §$P<0.005$ vs VGX+Hem+STIM.

eral cervical vagotomy did not significantly modify the rise in plasma TNF-α (190±24 pg/mL). Application of constant voltage pulses to the caudal vagus ends significantly reduced plasma TNF-α (87±15 pg/mL; t value=12.831; F value=132; $P<0.005$; Figure 4). Chlorisondamine pretreatment reverted the effects of vagal stimulation on plasma TNF-α (160±20 pg/mL; t value=9.094; F value=85.26; $P<0.005$; Figure 4).

Effects of Vagus Nerve Stimulation on Cardiovascular Measurements

MAP (36±2 mm Hg) and pulse pressure (PP; 15±1 mm Hg) were markedly impaired at the end of bleeding (Table 1). Application of constant voltage pulses to the caudal vagus

Figure 4. Plasma levels of TNF-α. Rats underwent hemorrhagic shock (Hem) or sham shock (Sham Hem). Hem shock animals were either vagotomized (VGX+Hem) or sham vagotomized (Sham VGX+Hem). Hemorrhaged animals subjected to vagotomy underwent the application of constant voltage pulses to the caudal vagus ends (STIM) or a sham stimulation (Sham STIM). Chlorisondamine (0.125 mg/kg) was injected subcutaneously 2 minutes before the start of bleeding. Each point represents the mean±SD of 10 animals. *$P<0.005$ vs Sham VGX+Hem; #$P<0.01$ vs VGX+Hem+Sham STIM; §$P<0.005$ vs VGX+Hem+STIM.

ends significantly improved (Table 1) MAP (66±5 mm Hg; t value=16.465; F value=310.34; $P<0.005$) and PP (39±3 mm Hg; t value=22.678; F value=576; $P<0.005$). Pretreatment with chlorisondamine (Table 1) reverted the effects of vagal stimulation on MAP (33±3 mm Hg; t value=18.111; F value=320.29; $P<0.005$) and PP (14±1 mm Hg; t value=23.623; F value=625; $P<0.005$).

Effects of Vagus Nerve Stimulation on Survival Rate and Survival Time

Table 2 shows the effect of acute hypovolemic shock on survival rate and survival time. Of 10 rats, none survived for 40 minutes after bleeding. Bilateral vagotomy did not significantly modify both survival time and rate. Application of constant voltage pulses to the caudal vagus ends significantly increased survival rate and time in shocked rats compared with those subjected to a sham stimulation. In these animals, the mean survival time was >180 minutes (t value=67.999; F value=>5000), and 3-hour survival rate was 100% (Table 2). Pretreatment with chlorisondamine (Table 2) prevented the protective effect of vagal stimulation on survival rate and time (33±6 minutes; t value=70.394; F value=6000; $P<0.005$).

Discussion

NF-κB modulates gene expression in various situations that require rapid and sensitive immune and inflammatory response.[3–6] Inactive NF-κB is present in the cytoplasm complexed with the inhibitory protein IκBα. NF-κB is activated by several incoming signals from the cell surface. Released from IκBα inhibition, NF-κB translocates into the nucleus and binds to κB motif of the target gene, in turn causing activation of several factors (cell adhesion molecules and cytokines) involved in the inflammatory response.[18]

NF-κB is involved in inflammatory gene activation in lung mononuclear cells during hemorrhage or hemorrhage resuscitation, and reactive oxidative intermediates induce NF-κB

TABLE 1.   MAP and PP in Rats Subjected to Hemorrhagic Shock (Hem) or Sham Shock (Sham Hem)

Treatment	Basal		Bleeding End		STIM End	
	MAP, mm Hg	PP, mm Hg	MAP, mm Hg	PP, mm Hg	MAP, mm Hg	PP, mm Hg
Sham Hem	96±4	42±4	98±5	43±3	92±6	41±4
Sham VGX+Hem	95±7	40±3	38±2	16±1	36±2	15±1
VGX+Hem+Sham STIM	90±8	38±3	40±3	14±1	33±3	14±1
VGX+Hem+STIM	91±6	40±3	38±2	15±2	66±5*†	39±3*†
Chlorisondamine+VGX+Hem+STIM	95±5	42±3	39±2	16±2	33±3‡	14±1‡

Hem shock animals were either vagotomized (VGX+Hem) or sham vagotomized (Sham VGX+Hem). Hemorrhaged animal subjected to vagotomy underwent an electrical stimulation (STIM) or a sham stimulation (Sham STIM).

Chlorisondamine (0.125 mg/kg) was injected subcutaneously 2 minutes before the start of bleeding. Each point represents the mean±SD of 10 animals.

*$P<0.005$ vs Sham VGX+Hem; †$P<0.005$ vs VGX+Hem+Sham STIM; ‡$P<0.005$ vs VGX+Hem+STIM.

translocation.[19] In this model, NF-κB required at least 1 hour after the hemorrhage procedure to be activated, and furthermore the mortality of this experimental models of shock was very low.

Acute hypovolemic hemorrhagic shock is a lethal type of shock[5,6,14–16] and is characterized by severe hypotension, increased levels of TNF-α, and vascular failure attributable to the loss of vascular reactivity after stimulation with vasoconstrictor stimuli. The inflammatory cytokine seems to play a pivotal role in the pathogenesis of this experimental model of circulatory shock, because anti–TNF-α monoclonal antibodies increase significantly survival, improve hypotension, and restore the impairment in vascular reactivity.[20] However, the mechanisms causing the systemic production of the inflammatory cytokine in this rapid and short-lasting hemorrhagic shock model remain to be fully elucidated.

Peak NF-κB activation and reduction of IκBα protein levels occur within 15 to 20 minutes after the end of bleeding,[6] thus confirming that NF-κB represents a rapid and early signal mechanism for controlling gene expression.

Several incoming signals may activate NF-κB,[18,21] and bacterial lipopolysaccharide is known to activate complexes of proteins that bind to NF-κB–regulatory DNA sequences.[21]

The oxidative state of the cell has been shown to influence the induction of NF-κB.[18,22] Reactive oxidative intermediates probably induce IκB phosphorylation by influencing the activity of tyrosine kinases. Previous experimental evidence from our laboratory has shown that in acute hemorrhagic shock, oxidative stress primes the activation of NF-κB in the liver, thereby leading to an inflammatory cascade that plays a pivotal role in the pathogenesis of this type of experimental shock.[6]

The central nervous system modulates the inflammatory response under several stressful conditions through humoral mechanisms.[7–12] Afferent vagus nerve fiber activation by endotoxin or cytokines stimulates hypothalamic-pituitary-adrenal-antiinflammatory response. However, a parasympathetic antiinflammatory pathway has been recently identified by which the brain modulates systemic responses in endotoxin shock.[13,23,24] This cholinergic antiinflammatory path-

TABLE 2.   Survival Time and Survival Rate in Rats Subjected to Hemorrhagic Shock (Hem) or Sham Shock (Sham Hem)

Treatment	Survival Time, min	Survival Rate		
		60 Minutes	120 Minutes	180 Minutes
Sham Hem	>180	10 of 10	10 of 10	10 of 10
Sham VGX+Hem	31±8	0 of 10	0 of 10	0 of 10
VGX+Hem+Sham STIM	38±3	0 of 10	0 of 10	0 of 10
VGX+Hem+STIM	>180*†	10 of 10*†	10 of 10*†	10 of 10*†
Chlorisondamine+VGX+Hem+STIM	33±6‡	0 of 10‡	0 of 10‡	0 of 10‡

Hem shock animals were either vagotomized (VGX+Hem) or sham vagotomized (Sham VGX+Hem). Hemorrhaged animal subjected to vagotomy underwent an electrical stimulation (STIM) or a sham stimulation (Sham STIM).

Chlorisondamine (0.125 mg/kg) was injected subcutaneously 2 minutes before the start of bleeding. Each point of survival time represents the mean±SD of 10 animals.

*$P<0.005$ vs Sham VGX+Hem; †$P<0.005$ vs VGX+Hem+Sham STIM; ‡$P<0.005$ vs VGX+Hem+STIM.

way seems to be mediated by nicotinic receptors in macrophages.[13]

We hypothesized that the cholinergic antiinflammatory pathway could be operative also in acute hypovolemic hemorrhagic shock and might serve as a suppressor mechanism to counterbalance liver NF-κB activation. To confirm this hypothesis, we investigated liver NF-κB activation and the consequent inflammatory cascade in vagotomized animals subjected to electrical stimulation of the caudal vagus ends.

Bilateral vagotomy slightly, but not in a statistically significant manner, increased NF-κB activation and the production of TNF-α. Electrical stimulation of the efferent vagal fibers significantly blunted NF-κB activation, decreased the mRNA for TNF-α, and reduced the circulating levels of the cytokine. Furthermore, the pretreatment with chlorisondamine, a peripheral nicotinic receptor–blocking agent,[17] reverted the protective effects of vagus nerve stimulation. Therefore, we hypothesize that acetylcholine, released by vagal ends, diffuses in liver resident macrophages before being completely hydrolyzed by serum acetylcholinesterases and blunts the NF-κB–triggered inflammatory cascade. This antishock mechanism seems also to influence the cardiovascular apparatus and the overall survival of the animals; in fact, vagal nerve stimulation improved MAP and PP and increased both survival time and rate.

Chlorisondamine is a general autonomic ganglion-blocking agent and antagonizes transmission at the level of either parasympathetic or sympathetic ganglia. However, in our experiments, we stimulated efferent vagal fibers, and chlorisondamine prevented the effect of such vagus nerve stimulation; therefore, it is reasonable to state that, under theses experimental conditions, chlorisondamine effects are a consequence of parasympathetic neurotransmission blockade.

Furthermore, the possibility that electrical stimulation effects of vagus nerve on liver NF-κB activation may be an indirect consequence of antiinflammatory hormones or cytokine release can be easily ruled out. In fact, it has been previously shown[13] that electrical stimulation of the distal ends of the divided vagus nerve does not stimulate any increase in either corticosteroids or interleukin-10.

Therefore, the present data confirm the importance of the cholinergic antiinflammatory pathway in the defense mechanisms of vertebrates and for the first time identify a previously unrecognized crucial role of this pathway in the modulation of the inflammatory cascade and in the activation of the transcription factor NF-κB in an acute model of hemorrhagic shock. Finally, these data would suggest the future possible therapeutic role of agents capable of stimulating/activating efferent vagal fibers or peripheral nicotinic receptors for the management of hemorrhagic shock.

Acknowledgments

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