Anesthesia-specific protection from endotoxic shock is not mediated through the vagus nerve

Joseph M. Fuentes, MD, Eric J. Hanly, MD, Alexander R. Aurora, MD, Antonio De Maio, PhD, *and* **Mark A. Talamini, MD,** *Baltimore, Md*

Background. *We have shown recently that volatile anesthetics significantly decrease inflammatory cytokine production and dramatically increase survival among rodents challenged with lipopolysaccharide (LPS). Because acetylcholine's interaction with nicotine receptors on tissue macrophages during vagus nerve stimulation has been implicated in the modulation of LPS-stimulated tumor necrosis factor alpha (TNF-α) production, we hypothesized that the mechanism of anesthetic immunoprotection is mediated through the vagus nerve.*

Methods. *Male Sprague-Dawley rats underwent bilateral cervical vagotomy (n = 20) or sham operation (n = 6). Twenty-four hours postoperatively, vagotomized rats were randomized into 3 groups: LPS injection (V+LPS, n = 6), LPS injection followed by 60 minutes of isoflurane anesthesia (V+LPS+ISO, n = 7), or saline injection (V+S, n = 7). Sham animals were also given LPS (Sham+LPS). A sublethal dose of LPS (8 mg/kg) was used. Blood samples were collected via cardiac puncture 90 minutes after LPS or saline injection, and plasma was isolated for the measurement of cytokines by enzyme-linked immunosorbent assay. Statistical differences between groups were detected by 1-way analysis of variance.*

Results. *Serum TNF-α was reduced significantly and interleukin (IL)-6 was abrogated completely among V+LPS+ISO rats, compared with both V+LPS and Sham+LPS animals ($P \leq .05$ for all). In contrast, levels of the anti-inflammatory cytokine IL-10 were similar among all LPS groups.*

Conclusions. *Isoflurane anesthesia administered simultaneously with the injection of LPS decreases serum production of TNF-α and IL-6 despite bilateral transection of the vagus nerve. Isoflurane-mediated attenuation of proinflammatory cytokine production occurs via a mechanism other than modulation of vagal output. (Surgery 2005;138:766-71.)*

From The Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, Md

ANESTHESIA is a fundamental component of the thousands of surgical procedures performed every day around the world to provide an environment in which analgesia, unconsciousness, and decreased muscle tone are achieved. Though general anesthetics have been used for the properties described above since 1842 when ether was introduced,[1] many of their specific molecular mechanisms of action remain an enigma.[2,3] We recently described that general anesthetics also possess anti-inflammatory properties. When given within 30 minutes of a lethal dose of *Escherichia coli* lipopolysaccharide (LPS), anesthesia attenuates tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-10,[4] and increases survival in rodents.[5] Both the central and peripheral components of the nervous system are possible targets through which the anesthetic and immunomodulatory effects of general anesthetics may be mediated.

Increasing evidence suggests that both efferent and afferent vagus nerve fibers play an important role in modulating the inflammatory response. A variety of insults can activate the innate immune system such as trauma and endotoxin, which trigger a cascade that activates monocytes, macrophages, and other cells, which in turn release cytokines into

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Reprint requests: Mark A. Talamini, MD, The Johns Hopkins Hospital, Department of Surgery, 600 North Wolfe St, Blalock 665, Baltimore, MD 21287-4665. E-mail: talamini@jhmi.edu.

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the bloodstream. Activation of the afferent vagus nerve fibers by injury, endotoxin, or cytokines triggers the hypothalamic-pituitary axis.[6] In turn, this neuroendocrine system prevents a chaotic inflammatory response by inhibiting local cytokine production through the release of glucocorticoids, adrenaline, and noradrenaline (the so-called "fight or flight" response). Previous studies also have shown that the febrile response and other signs of endotoxin-mediated "sickness syndrome"[7] are abolished in vagotomized rats.[8] In these animals, pyrogenic signals from the periphery to the brain are interrupted because the afferent signal has been severed. The efferent vagus nerve fibers also have been implicated in modulating inflammation. Direct electrical stimulation of the peripheral vagus nerve before and after a lethal dose of endotoxin has been shown to inhibit hepatic TNF synthesis, attenuate serum TNF, and prevent development of shock in a murine endotoxin model.[9] The protection offered through electrical stimulation of the vagus nerve has been coined the "cholinergic anti-inflammatory pathway"—vagus nerve stimulation (efferent signal) causes tissue release of acetylcholine, which binds specifically to nicotinic α-7 subunit receptors on macrophages, which in turn inhibit cytokine production.[10,11] Other studies have shown that efferent vagal signals inhibit nuclear factor-κB during acute hypovolemic shock.[12] Thus communication through the vagus nerve between the central nervous system and the periphery is bidirectional with respect to maintenance of equilibrium during stress and inflammation.

While its mechanism of action remains poorly understood, isoflurane remains a popular volatile anesthetic for both clinicians and researchers because of its rapid induction and recovery properties and because it produces minimal cardiovascular and respiratory depression. One current hypothesis is that isoflurane acts through a nicotinic acetylcholine receptor pathway.[13] Therefore, we hypothesized that the mechanism of anesthesia-specific immunoprotection found in our previous work involves efferent vagal signals. Polytraumatic patients likely will be subjected to multiple operations. As anesthesia is a fundamental component of any operative procedure, it is important to understand the effect of anesthetics on the inflammatory process.

MATERIAL AND METHODS

General procedures. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing 250 to 350 g were housed in plastic cages in which standard chow and water were available ad libitum. Animals were acclimatized to their environment for 3 to 5 days after arrival and were then fasted for 16 hours before procedures. The animal housing environment maintained a 12-hour light/dark cycle, a temperature of 72°F, and a humidity ranging between 30% to 70%. All surgical procedures were performed under aseptic conditions. Before surgery, the rats were preanesthetized rapidly in a 15 × 15 inch glass jar with the use of vaporized isoflurane (IsoFlo; Abbot Laboratories, North Chicago, Ill). Spontaneously breathing anesthetized animals were placed supine and restrained with adhesive tape. Anesthesia was maintained by delivering 2.5% vaporized isoflurane through a nose cone. After completion of the experiment, all rats were euthanized via anesthetic overdose. All animal procedures were performed according to a protocol approved by the Johns Hopkins Medical Institution Animal Care and Use Committee (ACUC protocol #RA01M445).

Cervical vagotomy. Animals were subjected to bilateral cervical vagotomy (n = 20) or sham operation (n = 6). The cervical area of the rat was prepped with 70% isopropyl alcohol. A ventral cervical midline incision was performed; careful dissection with fine microsurgical instruments (Roboz Surgical Instruments, Rockville, Md) was carried out to reach both vagus trunks and minimize unnecessary injury to surrounding tissue and vessels. Once identified with the aid of an operating microscope (Olympus, Melville, NY), the vagus trunks were divided with Castroviejo microscissors between two 4-0 silk ligatures. In the sham-operated animals, the vagus trunks were identified but were not ligated or divided. The skin incision was closed with a simple running stitch. Heat lamps were used to prevent hypothermic stress during the perioperative period. Postoperatively, animals were housed in plastic cages in which water was available ad libitum and behavior was monitored.

Experimental groups. Twenty-four hours postoperatively, vagotomized rats were randomized into 3 groups (Fig 1): LPS injection (V+LPS, n = 6), LPS injection under 60 minutes of isoflurane anesthesia (V+LPS+ISO, n = 7), or saline injection (V+S, n = 7). The sham-operated animals also received an LPS injection (Sham+LPS). The LPS was *E coli* serotype 026:B6 (Sigma-Aldrich, St. Louis, Mo) injected intraperitoneally at a sublethal dose (8 mg/kg). Blood samples were collected via cardiac puncture 90 minutes after LPS or saline injection, and plasma was isolated for detection of cytokines via an enzyme-linked immunosorbent assay with the use of a commercially available kit (Biosource International Inc, Camarillo, Calif).

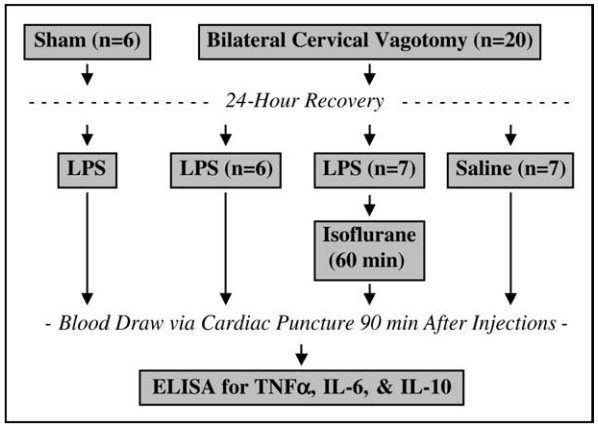

Fig 1. Schematic description of experimental design. *ELISA,* Enzyme-linked immunosorbent assay; *IL,* interleukin; *LPS,* lipopolysaccharide; *TNF-α,* tumor necrosis factor alpha.

Statistical analysis. Cytokine data were expressed as mean ± SEM. General differences in cytokine levels among all groups were determined with the use of 1-way analysis of variance (ANOVA); specific significances between groups were elucidated with multiple pairwise comparisons using the Student-Newman-Keuls method. Differences were considered significant at $P \leq 0.05$. Analysis was performed with Microsoft Excel (Microsoft Corp, Redmond, Wash) and Sigma Stat (SPSS, Inc, Chicago, Ill) software.

RESULTS

Cervical vagotomy model. The learning curve associated with performing cervical vagotomy plateaued quickly for our group. After 15 practice cases, the operators in our laboratory were very comfortable performing the procedure. Success for our group depended mainly on having the appropriate microinstruments, an operating microscope, and a bright fiber optic light source.

Isoflurane protection is not mediated through the vagus nerve. Serum TNF-α levels in response to LPS injection (Fig 2) were significantly lower among vagotomized animals who received isoflurane for 60 minutes after injection (V+LPS+ISO), compared with vagotomized animals who did not receive anesthesia (V+LPS, 86.9% reduction, $P \leq .05$) and compared with LPS-stimulated sham-operated animals (Sham+LPS, 93.6% reduction, $P \leq .05$). Furthermore, anesthesia completely abrogated the IL-6 response (100% reduction) to LPS in the V+LPS+ISO group ($P \leq .05$, compared with both V+LPS & Sham+LPS). IL-10 levels did not differ significantly between any of the LPS-stimulated groups. Vagotomized control animals who received saline instead of LPS did not develop detectible levels of any of the measured cytokines. While vagotomy alone did not affect the IL-6 response to LPS-stimulation (V+LPS vs. Sham+LPS), TNF-α levels in response to LPS were significantly

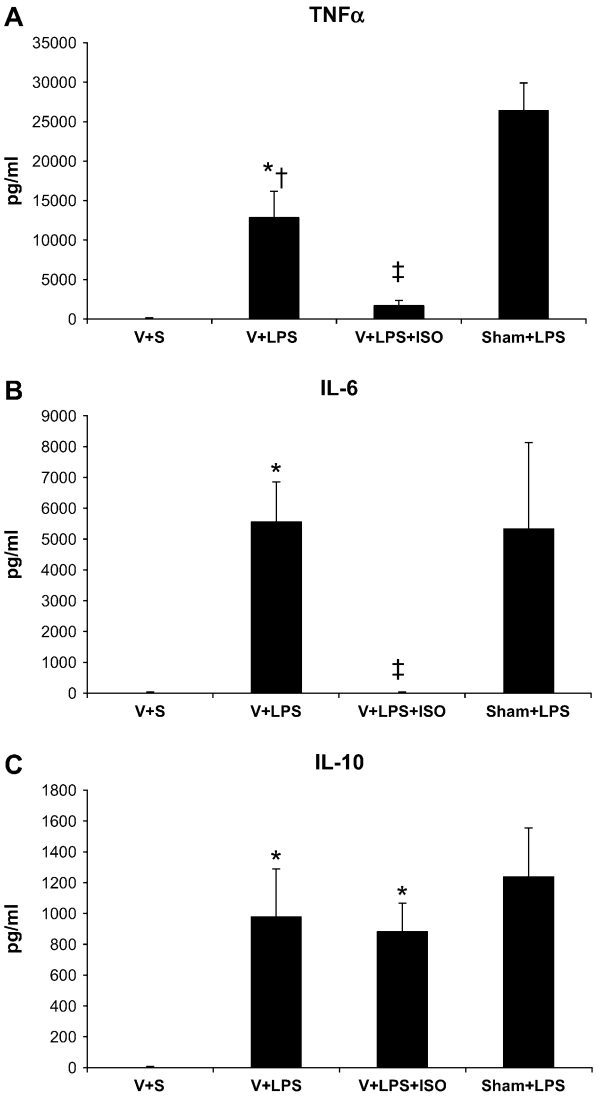

Fig 2. LPS-stimulated cytokine production in vagotomized rats. Rats treated with isoflurane (ISO) had a dramatic decrease in TNF-α and IL-6 production even after bilateral cervical vagotomy (V). Twenty-four hours after vagotomy or sham procedure, animals were injected intraperitoneally with lipopolysaccharide (**LPS**, 8 mg/kg) or saline (**S**). Ninety minutes after injection, blood was collected via cardiac puncture to measure serum TNF-α (**A**), IL-6 (**B**), and IL-10 (**C**) by enzyme-linked immunosorbent assay. Data are mean ± SEM. *$P \leq .05$ vs Sham+LPS & V+LPS; †$P \leq .05$ vs Sham+LPS; ‡$P \leq .05$ vs V+LPS+ISO.

lower in the V+LPS group, compared with the Sham+LPS group.

DISCUSSION

The concept of "sympathovagal balance," an important concept in surgery, has arisen to describe the complex autonomic state that results from the interaction between sympathetic and vagal influences.[14] Because we recently have demonstrated that anesthetics have a profound effect on the outcome from LPS-induced sepsis, both in terms of reductions in serum markers[4] of inflammation and increases in survival,[5] this concept led us to elucidate whether or not anesthetics exerted their anti-inflammatory effects via the cholinergic anti-inflammatory pathway. However, in the current study we have shown that isoflurane anesthesia significantly attenuates serum circulation of TNF-α and completely abolishes IL-6 even among animals who have undergone bilateral cervical vagotomy. These data suggest that the proinflammatory-attenuating effects of anesthesia are independent of the vagus nerve.

In contrast, serum expression of the anti-inflammatory cytokine IL-10 was no longer reduced among the vagotomized rats in our study. Activation of macrophage acetylcholine receptors has been shown to specifically inhibit the release of pro-inflammatory cytokines, but not anti-inflammatory cytokines.[9] Thus it is not surprising that the anesthesia-dependent variable expression of LPS-induced production of cytokines from different classes would behave differently in response to vagotomy. Therefore, it is conceivable that the effect of isoflurane on endotoxin-mediated IL-10 release is, in fact, mediated through the vagus nerve.

One aspect of our data that is at odds with findings from another group warrants discussion and relates to the effect of vagotomy alone (ie, without a long period of anesthesia after LPS administration) on the cytokine response to endotoxin. Borovikova et al[9] have published that vagotomized animals have a more vigorous serum TNF-α response to LPS than do sham-operated animals. However, in our study, the V+LPS group yielded significant lower TNF-α levels than the Sham-LPS group. The most likely explanation for this discrepancy relates to the necessarily different methodology between the studies. Because our study addresses the mechanism of anesthesia-specific immunoprotection and the execution of surgical vagotomy requires the application of a brief period of anesthesia, we felt it important to allow the animals in our study a day in which to recover from anesthesia before administering LPS with or without another prolonged period of anesthesia. In contrast, Borovikova's group performs vagotomy in the same setting as the administration of LPS. The absence of basal vagal stimulation for a 24-hour period may have a significant influence on the subsequent systemic response to LPS challenge. Moreover, in previous studies we have explored chemical vagotomy with the anticholinergic agent atropine in the presence of isoflurane anesthesia. Consistent with the current study, chemically vagotomized (atropine-treated) animals experience a decrease in TNF-α, compared with LPS control. Furthermore, just as in the current study, IL-6 levels are not affected by vagotomy alone. Finally, as in the present study, isoflurane anesthesia significantly decreases both TNF and IL-6 responses to LPS, compared with both sham and chemically vagotomized animals (unpublished data). These studies corroborate each other, suggesting that isoflurane-mediated attenuation of LPS-induced proinflammatory cytokine production is independent of the cholinergic pathway, including the vagus nerve.

One potential criticism of our current study was our exclusion of a sham-operated control group receiving both LPS and isoflurane. Because our previous work consistently has shown in multiple different experiments that such a group reduces proinflammatory cytokine levels by 80% to 90%, compared with LPS-challenged groups who did not receive isoflurane (reductions comparable to the reduction observed in the current study among the vagotomized animals who received both LPS and isoflurane),[4,15] we elected not to include this group in the current experiment.

The mechanism by which general anesthetics exert their protective immunomodulatory effects remains uncertain. Multiple ischemia-reperfusion models have been developed on the basis of the original description by Murry et al[16] that myocardial ischemic preconditioning decreases the injury produced by a subsequent ischemia-reperfusion insult. Several volatile anesthetics, including isoflurane, also have been shown to be cardioprotective through a mechanism similar to ischemic preconditioning.[17] Data suggest that anesthetic-induced preconditioning occurs via the activation of potassium adenosine triphosphate channels,[18] which has been shown to inhibit apoptosis and necrosis.[19] Alternatively, evidence implicates the release of small quantities of reactive oxygen species in the mechanism of anesthetic-induced preconditioning.[20,21] Furthermore, a number of studies have shown that anesthetic-induced preconditioning also inhibits lipopolysaccharide-induced inflammation in endothelial tissue and vascular smooth muscle cells.[22,23]

Our previous work is consistent with the concept that anesthesia is protective via preconditioning. We have shown that anesthesia pretreatment inhibits the production of proinflammatory cytokines and increases survival among LPS-challenged rodents.[15] The effect appears to be greatest when the anesthesia is administered before or simultaneous to the LPS challenge and when the duration of anesthesia is the longest. Finally, these findings appear to be consistent over a broad range of sedative and anesthetic classes.[4] Anesthetic-induced preconditioning appears to be the common denominator in all of these findings. One might be concerned that the brief (10-18 minutes) period of anesthesia required to perform vagotomy or sham operation the day before the actual experiment in the current study might, by itself, establish a preconditioned state. However, our previous work has shown that this limited duration of anesthesia does not influence the cytokine response to LPS.[15] Furthermore, because data from other researchers demonstrate that a range of at least 25 to 45 minutes of isoflurane anesthesia is necessary for ischemia preconditioning to occur,[24] we did not feel it necessary to perform additional experiments at time points later than 24 hours after limited exposure to anesthesia.

The fact that many different anesthetic agents appear to protect against endotoxic shock implies that the phenomenon may be due to a more general physiologic change that takes place during anesthesia. Our data could be explained by a reduction in systemic blood flow, which slows LPS delivery to the liver and allows for more effective LPS clearance. Alternatively, global anesthesia-induced physiologic changes in hormonal or other functions may modify indirectly the inflammatory response or regulate the explosive inflammatory cascade.

CONCLUSION

The volatile anesthetic isoflurane decreases the TNF-α and IL-6 response to a sublethal dose of LPS even among animals having undergone bilateral cervical vagotomy. In contrast, isoflurane loses its attenuating effect on IL-10 in vagotomized rats. These data indicate that a pathway that is not vagally mediated is responsible for the mechanism of isoflurane-induced proinflammatory cytokine inhibition. Further studies should attempt to elucidate the specific mechanism involved in this phenomenon, explore the specific effects of vagotomy on anesthesia-mediated reduction of anti-inflammatory cytokine production, and further investigate the precise role that general anesthetics might play as adjunct therapeutic agents for septic patients.

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