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Vagus nerve stimulation reduces infarct size in rat focal cerebral ischemia: An unlikely role for cerebral blood flow

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Abstract

We sought to investigate the effect of cervical vagus nerve stimulation (VNS) on cerebral blood flow (CBF), infarct volume, and clinical outcome in a model of middle cerebral artery occlusion in rats. Electrical stimulation of the right and left vagus nerves was initiated 30 min after the induction of the right-sided ischemia and lasted for 1 h. Infarct size measurement revealed that the volume of ischemic damage was 41–45% smaller in animals receiving stimulation as compared with control animals. Both the right and left VNS caused subtle reduction in CBF during each 30-s stimulation period that quickly returned back to the baseline level at the end of each stimulation cycle. There was no significant effect of VNS on CBF during the entire 1-h stimulation period. The effect of VNS on tissue outcome was associated with better neurological outcome at both 1- and 3-day time points after the induction of ischemia. These findings suggest that VNS-induced protection against acute ischemic brain injury is not primarily mediated by changes in CBF, stimulation of both the right and left nerve have comparable effects, and VNS is effective after ipsilateral and contralateral focal ischemia.

Keywords

Cerebral ischemia; Vagus nerve; Cerebral blood flow; Electrical stimulation; Rat

1. Introduction

Electrical stimulation of the left cervical vagus nerve is an approved treatment by the FDA as an adjunctive therapy for partial epilepsy and drug-resistant depression and has been used clinically since 1997. We have recently shown that the right-sided vagus nerve stimulation (VNS) initiated 30 min after an ipsilateral transient middle cerebral artery (MCA) occlusion reduces infarct volume by approximately 50% in rat (Ay et al., 2009). The mechanism by which VNS reduces infarct size is not known. It has been suggested that VNS causes cerebral vasodilation and increased cerebral blood flow (CBF) under non-ischemic conditions (Nakai et al., 1982; Nakai et al., 1993). Positron emission tomography (PET) and functional MRI (fMRI) studies in patients with epilepsy and depression have demonstrated that VNS augments CBF in certain brain regions including bilateral frontal and prefrontal cortices, thalamus, and right anterior insula (Conway et al., 2006; Henry et al., 1998; Henry et al., 1999; Henry et al., 2004; Lomarev et al., 2002). Because compromise of blood flow is

the key event in the pathophysiology of ischemic brain injury, augmentation of CBF is certainly desirable.

In the present study, we sought to reproduce our earlier findings on the effect of VNS on tissue outcome by the left-sided VNS and explore whether infarct reducing effect of VNS is mediated by an increase in CBF.

2. Results

There was no difference in body temperature, arterial blood gases, and pH between control and treatment groups. Three animals (all in the left VNS protocol; one in the stimulation group and two in the control group) died during electrode implantation, most likely due to cardiac arrest. However, mortality rate during the survival period in both protocols was less than 10%.

Transient occlusion of the right MCA resulted in infarct in the ipsilateral cerebral cortex and underlying striatum. In the right-VNS protocol, the mean infarct volume and SEM was $43.0 \pm 3.9\%$ ($n = 8$) of the contralateral hemispheric volume in untreated animals. This was significantly larger than the mean infarct volume in VNS-treated animals ($23.9 \pm 1.2\%$, $n = 8$; unpaired t -test: $t = 2.306$, $p = 0.0016$; Fig. 1A). In the left-VNS protocol, the mean infarct volume of the contralateral hemispheric volume in untreated animals was also significantly larger than the mean infarct volume in VNS-treated animals ($43.1 \pm 1.7\%$ vs. $25.4 \pm 0.8\%$, $n = 8$ in each group; unpaired t -test: $t = 9.645$, $p < 0.0001$; Fig. 1B).

VNS-treated animals had better neurological scores 24 h and 3 days after ischemia compared to control animals (Fig. 1C and D). In the right-VNS protocol, the neurological score was 2.9 ± 0.1 at 3 h and 2.9 ± 0.1 at 24 h after ischemia in the control group ($n = 8$ at both time points). The corresponding scores in VNS-treated animals were 2.9 ± 0.1 and 2.4 ± 0.2 , respectively ($n = 8$ at both time points). The difference was significant at 24 h after ischemia (repeated measures ANOVA: $F(1,14) = 5.600$, $p = 0.0329$; Mann-Whitney U test: $p = 0.0225$). In the left-VNS protocol, the neurological score was 2.9 ± 0.1 at 3 h and 3.1 ± 0.1 at 3 days after ischemia in the control group ($n = 8$ at both time points). The corresponding scores in VNS-treated animals were 2.9 ± 0.1 and 2.2 ± 0.1 , respectively ($n = 8$ at both time points). The difference was significant at 3 days after ischemia (repeated measures ANOVA: $F(1,14) = 18.976$, $p = 0.0007$; Mann-Whitney U test: $p = 0.0010$).

Electrical stimulation of both cervical vagus nerves caused an immediate and transient decrease in systolic and diastolic blood pressure, heart rate (HR), and regional CBF (rCBF) (Table 1). Stimulation-induced reduction in these parameters lasted for 30 s and completely returned back to the baseline level at the end of each stimulation cycle (Fig. 2). There was no difference in the magnitude of this effect between the right and the left VNS (Table 1). An assessment for the entire period of stimulation did not reveal a significant difference in mean arterial blood pressure (MABP; Fig. 3A and B) and HR by the VNS as compared with the control group.

MCA occlusion caused more than 60% decrease in laser Doppler signal and this was almost completely reversed by reperfusion in both groups (Fig. 3C and D). Analysis of rCBF before and after each VNS treatment cycle as well as during the entire treatment period (excluding the actual stimulation period) revealed that VNS had no overall effect on CBF in both the right-VNS protocol (repeated measures of ANOVA: $F(1,14) = 0.008$, $p = 0.9282$) and the left-VNS protocol (repeated measures of ANOVA: $F(1,14) = 0.027$, $p = 0.8710$). However, there was a slight, non-significant increase in CBF in the right VNS-treated animals as compared to controls during the entire treatment period (Fig. 3C). On the other hand, during delivery of electrical stimulation, there was a transient drop in CBF synchronized with the

decrease observed in MABP and HR in both experimental protocols (Fig. 2C and D; Table 1).

3. Discussion

The present study confirms our prior findings that the right VNS, initiated within 30 min after the onset of an ipsilateral transient MCA occlusion, reduces infarct volume by approximately 50% in rats (Ay et al., 2009) and provides new information in multiple domains. First, it demonstrates that the stimulation of the left vagus nerve is also as effective as the right side in reducing the infarct volume. Second, stimulation of vagus nerve exerts infarct reducing effect in the contralateral hemisphere. This finding is in accordance with data from studies in humans showing that unilateral VNS leads to alterations in both hemispheres (Henry et al., 1999). Third, the effect of VNS on tissue outcome is associated with significant and persistent improvement in clinical outcome; the neurological scores were significantly better when measured 1 and 3 days after the right MCA occlusion in VNS-treated animals as compared to controls. Lastly, we demonstrate that VNS-mediated reduction in infarct volume is not primarily mediated by increase in CBF in the ischemic region. On the contrary, we observed transient and modest reduction in CBF that was perfectly coupled with MABP and HR changes during each 30-s stimulation period. There was no overall effect of either the right or the left VNS on CBF during the entire period of treatment. Although studies by PET (Conway et al., 2006; Henry et al., 1998; Henry et al., 1999; Ko et al., 1996) and BOLD-fMRI (Lomarev et al., 2002) have demonstrated that stimulation of the cervical vagus nerve increases CBF in several parts of the brain in patients with epilepsy or depression, there is currently no published data examining VNS-induced CBF changes under ischemic conditions. Because both PET and fMRI measure changes in tissue perfusion and oxygen metabolism primarily during neural activation, it is possible that VNS does not lead to primary dilation of cerebral arteries but rather the observed increase in CBF in functional studies is secondary to cortical activation by VNS.

Although cerebrovascular system is innervated by the parasympathetic system, this innervation is not provided by the vagus nerve. Branches of the facial nerve that originate from the superior salivatory nucleus in the pons innervate the cranial vasculature (Nakai et al., 1993). Nevertheless the medullary vagal complex, particularly the solitary nucleus where the primary activation following VNS occurs, has connections with other brain regions that are known to potentially alter the vascular tone upon stimulation (Agassandian et al., 2003; Walters et al., 1986). Some of the important connections of solitary nucleus include superior salivatory nucleus, fastigial nucleus, and locus coeruleus (Agassandian et al., 2003; Morgane and Jacobs, 1979). Selective stimulation of some of these structures or their anatomical extensions is known to cause cerebral vasodilation and increased CBF under non-ischemic conditions (Nakai et al., 1982; Nakai et al., 1993). Our findings suggest that VNS fails to augment CBF under ischemic conditions. This could be due to reduced vasodilatory capacity of cerebral arteries during cerebral ischemia as a result of exhausted autoregulatory vasodilation to maintain tissue perfusion (Derdeyn et al., 2002). Therefore, subtle VNS-induced CBF changes may escape from detection. It is also possible that, as previously suggested (Henninger and Fisher, 2007), isoflurane used for anesthesia is a potent vasodilator and this might have mitigated a potential effect of VNS on CBF as well (Cucchiara et al., 1974).

It may be valuable to explore alternative or blood flow-independent mechanisms for VNS-mediated protection against ischemic injury. VNS is a very potent inhibitor of inflammation; it inhibits cytokine synthesis by activating alpha-7 nicotinic acetylcholine receptors on macrophages (Borovikova et al., 2000; Wang et al., 2003). This avoids tissue from further injury under ischemic conditions. It has been shown that VNS protects against myocardial

ischemia in rats and this effect depends on inhibition of cytokine synthesis through activation of $\alpha 7$ subunit of the nicotinic acetylcholine receptors (Mioni et al., 2005; Tracey, 2007). Future studies are needed to test the immune-modulation hypothesis by VNS in cerebral ischemia. VNS is also a powerful inhibitor of neuronal excitability (Krahl et al., 1998); it increases GABA levels and GABA receptor density in patients with epilepsy (Ben-Menachem et al., 1995; Marrosu et al., 2003). Increased GABAergic activity, in turn, correlates with favorable tissue outcome in cerebral ischemia (Lyden and Hedges, 1992). Finally, VNS may exert neuroprotection through activation of structures connected to the medullary vagal complex. For instance, direct electrical stimulation of fastigial nucleus causes inhibition of neuronal excitability and expression of several genes involved in suppression of inflammatory reaction and apoptotic cascade, and thus ameliorates ischemic brain injury (Golanov and Zhou, 2003; Reis and Golanov, 1997).

This study is subject to a number of limitations. We used laser Doppler flowmetry for CBF measurement. Doppler flowmetry provides continuous, real-time measurement of CBF. Its high temporal resolution allowed us to continuously monitor CBF during and in between stimulations. Nevertheless, Doppler flowmetry does not provide perfusion changes in absolute values and offers only limited spatial information when performed at a single point (Royl et al., 2006). Marginal increases in rCBF in regions that remain outside the sampling volume may have a biologically significant effect in critically perfused tissue that is still alive. Further studies with multi-point laser Doppler flowmetry, or preferentially with techniques with higher spatial resolution such as perfusion MR imaging are necessary in order to reject blood flow hypothesis definitely. Since we did not suppress anterograde propagation of pulses generated by electrical stimulation on vagus nerve (anodal block), there was a decrease in blood pressure and HR during stimulation. This inadvertently affected CBF; the observed decreases in CBF temporally overlapped with the pulse train in each stimulation cycle, suggesting that CBF decreases were likely secondary to the reduction in blood pressure. It remains to be studied whether anodal block unmasks a potential effect of VNS on CBF and further augments its neuroprotective effect in cerebral ischemia.

Several approaches including hypothermia, pharmacologic neuroprotection, induced hypertension, and delayed recanalization/reperfusion have been attempted to protect or salvage ischemic brain tissue yet none has been proven to be effective in humans despite theoretical appeal and success in several animal models (Fisher and Bastan, 2008). All these approaches are systemic, attempting to circumvent or override the intrinsic autoregulatory system that the brain has. Modulation of endogenous mechanisms through electrical stimulation of autonomic nerves for the treatment of stroke is a novel direction with only a few—albeit very promising—studies performed to date (Henninger and Fisher, 2007; Masada et al., 1996; Reis et al., 1991). Our data suggest that VNS modulates the endogenous blood flow-independent mechanisms to salvage the tissue at risk for infarction. Future studies are needed to understand the mechanism that VNS ameliorates the amount of tissue that undergoes infarction. Such studies will also be critical to develop new stimulation techniques that are also feasible in humans.

4. Experimental procedures

All experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Adult male Wistar rats (350–400 g, Charles River Laboratories, Wilmington, MA) were anesthetized by isoflurane (4–5% for induction, 1–2% for maintenance; in 30% oxygen 70% nitrous oxide) and were kept under anesthesia throughout the experimental period. Rectal temperature was intermittently measured and

maintained at 37.5 °C. A burr hole was drilled over the right parietal cortex (5 mm lateral and 1mm posterior to bregma) for the positioning of the laser Doppler flowmeter probe. Previous studies suggest that this location corresponds to a transitional zone in the MCA territory between severely ischemic and non-ischemic tissues (Nakai et al., 1997; Selman et al., 2004). rCBF was measured starting before ischemia until early reperfusion (Blood Flow Meter, ADInstruments, Colorado Springs, CO). Care was taken to avoid vibration to prevent artifacts in laser Doppler recordings. With this method, it is possible to measure changes within a tissue volume of approximately 1 mm³. The right femoral artery was cannulated to monitor arterial blood pressure, HR, blood gases and pH. Cerebral ischemia was produced by intraarterial filament (diameter: 0.39 ± 0.02 mm; Doccol Corporation, Redlands, CA) occlusion of the right MCA for 105 or 120 min followed by reperfusion (Longa et al., 1989). Self-constructed VNS electrodes were implanted on the right or the left cervical vagus nerve, cathode cranial–anode caudal (Smith et al., 2005).

There were two experimental protocols: (1) the right-VNS protocol: stimulation electrodes were implanted into the right cervical vagus nerve in both control or treatment groups and animals were sacrificed 24 h after 120 min ipsilateral ischemia followed by reperfusion; (2) the left-VNS protocol: stimulation electrodes were implanted into the left cervical vagus nerve in both control or treatment groups and animals were sacrificed 3 days after the contralateral 105 min ischemia followed by reperfusion (in our preliminary studies we found that the survival of animals for 3 days was improved after reducing the duration of ischemic insult from 120 to 105 min without causing a prominent change in infarct volume). Animals were assigned to the treatment or control groups by computer-generated random sequence. The sample size calculation (n=8) was based on a 40% anticipated difference in infarct volume by treatment at a power of 90% at an alpha level of 0.05.

In animals receiving VNS, stimulation was initiated 30 min after the induction of ischemia. Square pulses were delivered using Grass Model S48 stimulator and constant current unit (Grass Instruments, West Warwick, RI) at 0.5 mA, 30 s train of 0.5 ms pulses delivered at 20 Hz (Ay et al., 2009; Smith et al., 2005). Electrical stimulation was repeated at every 5 min for 1 h in VNS-treated animals. In the control animals, all procedures were duplicated including implantation of electrodes on the vagus nerve but no stimulus was delivered. At the end of the experimental period, stimulating electrodes and arterial catheters were removed and incisions were sutured. Before the incision and at the end of surgery 0.25% bupivacaine was topically applied to the wounds to alleviate surgical pain. Also, buprenorphine HCl (0.05 mg/kg; sc) was injected, animals were allowed to awaken. Neurological deficit was evaluated on a five-point scale (0 = no deficit to 4 = no spontaneous walking) (Bederson et al., 1986; Longa et al., 1989) 3 h after ischemia and just before the euthanasia (24 h after ischemia in the right VNS group and 3 days after ischemia in the left VNS group). Briefly, rats were (1) held by the tail, suspended on air, and observed for forelimb flexions; (2) placed on an underpad, held by the tail, and laterally pushed to slide the forelimbs on each side separately to observe resistance; (3) allowed to move freely to observe circling behavior.

Animals were killed by potassium chloride injection under isoflurane anesthesia and the brain was rapidly removed. Starting from the frontal, the brain was immediately sliced into seven 2 mm-thick sections using brain matrix and these were incubated with 2,3,5-triphenyltetrazolium chloride at room temperature for 30 min. The sections were then transferred into 10% formalin and kept at 4 °C for 48 h. Images of these sections were then obtained by a digital camera. Infarct area as well as ipsilateral non-infarct area and contralateral hemispheric area were outlined manually in all of the sections using Image J (NIH) in a blinded-fashion. Infarct volume was calculated by multiplying infarct area

(contralateral hemispheric area minus ipsilateral non-infarct area) by slice thickness and expressed as a percentage of contralateral hemispheric volume.

Data were expressed as mean $\pm$ SEM. Laser Doppler flowmeter measurements were expressed as percent of baseline. Physiological measurements [MABP, HR, blood gases and pH, and rectal temperature] were analyzed by repeated measures ANOVA followed by Student–Newman–Keuls test when needed. Infarct volumes were compared using unpaired *t*-test. Neurological scores were compared using repeated measures ANOVA followed by Mann–Whitney *U* test when needed. *p* Value of <0.05 was considered statistically significant.

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Fig. 1. The effect of the right (A, C) and left (B, D) VNS on infarct size and neurological outcome. Data were represented as mean $\pm$ SEM (A and B, $*p < 0.05$ by unpaired t -test) and median $\pm$ IQR (C and D, $*p < 0.05$ by Mann–Whitney U test).

Fig. 2. Representative recordings showing that electrical stimulation of the right (A and C) and left (B and D) vagus nerve caused a transient decrease in arterial blood pressure (A and B) and rCBF (C and D). Note that this effect was observed only during the stimulation period (about 30 s) and returned to the pre-stimulation level at the end of the stimulation.

Fig. 3.
Overall effect of the right (A and C) and left (B and D) VNS on MABP (A and B) and rCBF (C and D). Data were represented as mean $\pm$ SEM.

Table 1

Mean change by VNS in MABP, HR, and rCBF.

		Right-VNS	Left-VNS
MABP	mm Hg	18.7±6.3	20.3±3.5
	% reduction relative to baseline	16.6±6	17.2±3.2
HR	bpm	110±31.9	145.6±18.5
	% reduction relative to baseline	29.0±8.5	34.3±4.2
rCBF	perfusion unit	22.7±10.3	29.4±10.4
	% reduction relative to baseline	19.5±8.1	17.5±5.6