Microinjection of DLH into the region of the caudal ventral respiratory column in the cat: evidence for an endogenous cough-suppressant mechanism

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Poliacek I, Wen-Chi Corrie L, Wang C, Rose MJ, Bolser DC. Microinjection of DLH into the region of the caudal ventral respiratory column in the cat: evidence for an endogenous cough-suppressant mechanism. J Appl Physiol 102: 1014–1021, 2007. First published November 30, 2006; doi:10.1152/japplphysiol.00616.2006.—The caudal ventral respiratory column (cVRC) contains premotor expiratory neurons that play an important role in cough-related expiratory activity of chest wall and abdominal muscles. Microinjection of D,L-homocysteic acid (DLH) was used to test the hypothesis that local activation of cVRC neurons can suppress the cough reflex. DLH (20–50 mM, 10–30 nl) was injected into the region of cVRC in nine anesthetized spontaneously breathing cats. Repetitive coughing was elicited by mechanical stimulation of the intrathoracic airways. Electromyograms (EMG) were recorded bilaterally from inspiratory parasternal and expiratory transversus abdominis (ABD) and unilaterally from laryngeal posterior cricoarytenoid and thyroarytenoid muscles. Unilateral microinjection of DLH (1–1.5 nmol) elicited bilateral increases in tonic and phasic respiratory ABD EMG activity, and it altered the respiratory pattern and laryngeal motor activities. However, DLH also decreased cough frequency by 51 ± 7% compared with control (P < 0.001) and the amplitude of the contralateral (-35 ± 3%; P < 0.001) and ipsilateral (-34 ± 5%; P < 0.001) ABD EMGs during postinjection coughs compared with control. The cough alterations were much less pronounced after microinjection of a lower dose of DLH (0.34–0.8 nmol). No cough depression was observed after microinjections of vehicle. These results suggest that an endogenous cough suppressant neuronal network in the region of the cVRC may exist, and this network may be involved in the control of cough reflex excitability.

CONSIDERABLE PROGRESS HAS been made in the understanding of central cough mechanisms since the first evidence that respiratory neurons in the ventrolateral medulla participated in coughing (14, 21). Our current conceptualization of the neurogenesis of cough holds that there is a common respiratory and cough-generating neuronal network in the brain stem (38, 40, 41). However, this concept of cough generation cannot explain alterations of cough excitability induced by the administration of antitussive drugs (6, 7) or the elimination of the cough reflex by kainic acid lesions in several brain stem areas that are not involved in respiratory rhythm generation (22, 23, 35, 36). Our laboratory has proposed the existence of a functional element in the cough generation system that we have termed a gate (7). This excitatory element enables a reconfigured respiratory pattern generator to produce single or repetitive coughs and provides excitatory input to expiratory premotor neurons in the medulla (8). Whether the gating mechanism is the sole brain stem control element that regulates coughing or is itself modulated by other regulatory elements is unknown. Cough can be under inhibitory control. For example, this behavior can be inhibited voluntarily in awake humans (19). While it is presumed that this voluntary cough suppression is solely due to suprapontine pathways, the role of brain stem mechanisms in mediating this effect is unknown.

The medullary caudal ventral respiratory column (cVRC) is associated with the nucleus retroambigualis and contains a high number of expiratory modulated neurons. A great majority of these cells are premotor expiratory neurons (1, 27), which transmit motor drive to spinal expiratory motoneurons (24, 30) and so play an important role in numerous expiratory-related behaviors involving the chest wall and abdominal muscles such as cough (17, 31, 39). This population of expiratory neurons is thought to have few axon collaterals to other areas of the brain stem (1, 15). As such, this population of neurons is thought to be solely premotor and has not been considered important in respiratory rhythmicity (see also Refs. 18, 42, 45). However, earlier studies also revealed that injection of the excitatory amino acid agonist D,L-homocysteic acid (DLH) into the region of cVRC induced abdominal muscle activation (10, 51), increased laryngeal muscle activity and blood pressure (51), and decreased inspiratory neuronal and motor activity, which frequently led to the complete apnea (10). Bongianni et al. (10) proposed that axon collaterals to the other areas of the brain stem from the population of cVRC premotoneurons were more common than previously thought, and as such this population of neurons could account for the effects that they observed. This group also proposed an alternative hypothesis that other neurons in or near the cVRC were stimulated by the DLH microinjections and that they were responsible for the apnea produced by the intervention. Apnea can also be associated with inhibition of cough (47). We speculated that chemical excitation of neurons in the region of the cVRC would result in suppression of cough. Alternatively, if no population of neurons exists in this area that participates in the inhibition of cough, chemical activation of the expiratory premotor pathway in the cVRC should only increase cough-related expiratory spinal motor activity with no other alteration in the behavior.

METHODS

Experiments were performed on 12 female cats (3.5 ± 0.2 kg). The animals were anesthetized with pentobarbital sodium (35 mg/kg iv).

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Mechanical stimulation of the intrathoracic airways elicited stable repetitive coughing with an average of 6.8 ± 1.4 coughs per 10-s stimulation in the control period. Five animals received only DLH microinjections, three animals received only microinjections of aCSF (27–60 nl, 37 ± 3 nl), and four animals received microinjections of both aCSF and DLH. Of the nine animals that received DLH, five were injected only with a higher dose (50 mM, 20–30 nl, 1.3 ± 0.2 nmol) of this EAA agonist. Two cats received only a smaller total dose of DLH (20 or 50 mM, 10–40 nl, 0.5 ± 0.2 nmol), and two animals received both doses of DLH.

DLH microinjection (1.3 ± 0.2 nmol, range 1–1.5 nmol, 11 injections, 7 locations, 7 cats) in or near the region of the cVRC led to the suppression of tracheobronchial cough induced by mechanical stimulation (Fig. 1). The microinjection induced a transient increase of ABD EMG activity (Fig. 1) and significantly reduced cough number by 51 ± 7% (P < 0.001) in postinjection trials compared with control (Fig. 2). This inter-
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vention also reduced the amplitudes of expiratory-related cough ABD EMG activity in both the contralateral and ipsilateral sides by 35 ± 3% (P < 0.001) and by 34 ± 5% (P < 0.001), respectively (Fig. 2). The cough suppression lasted 10–15 min after the injection (Fig. 2). There was no significant effect of DLH injection on PS EMG amplitudes during cough (Fig. 2); however, there was a high degree of variability in the cough PS EMG responses after DLH injection with cough-related PS EMG responses markedly suppressed in some trials (Fig. 1). In four cats when lower doses of DLH (0.5 ± 0.2 nmol, range 0.34–0.8 nmol, 4 injections, 4 locations) were injected into the area of cVRC, there was a reduction in the amplitudes of cough ABD bursting activity in the range 17–25% for both the ipsi- and contralateral sides in the post-DLH periods. This inhibitory effect was statistically significant (P < 0.05) only for the ABD EMG contralateral to the injection site. The number of coughs was not significantly altered under this condition.

PCA and ThAr activities were recorded during coughing in five cats injected with higher dose of DLH (1–1.5 nmol). There were no significant differences in laryngeal PCA or ThAr EMG amplitudes in any of four laryngeal phases of cough reflex (Table 1), even though the cough ABD EMG amplitudes were significantly reduced after the DLH injection (Fig. 2).

To determine the mechanism by which cough number decreased in response to DLH microinjection, we analyzed the effect of this intervention on cough phase durations (Table 2). Even though cough number was reduced by 51% (Fig. 2), there was no significant impact of DLH microinjection on the total cough cycle time (CTtot), CT1, or CTTE (Table 2) even when these durations were normalized to the control CTtot (expressed as percentages of the control CTtot). Similarly, there were no significant alterations in the duration of ABD EMG activity during the cough expiratory phase caused by DLH microinjections (0.83 ± 0.08 s compared with the control 0.82 ± 0.09 s).

The control cough parameters in the aCSF-treated group of animals (n = 7; cough number per 10-s stimulation 4.8 ± 0.9; amplitudes of contralateral and ipsilateral ABD EMGs 90 ± 10% and 93 ± 9%, respectively; amplitudes of contralateral and ipsilateral PS EMG 93 ± 10% and 98 ± 11%, respectively) were not significantly different from those measured during the control period in DLH-treated cats. Microinjections of the aCSF into the area of cVRC (13 microinjections, 8 locations) had no significant influence on number of coughs (4.5 ± 0.9) or any other parameter of the cough reflex induced by mechanical stimulation of the intrathoracic airways.

Microinjection of DLH in the vicinity of the nucleus retroambiguus produced increases in ABD EMG activity (Fig. 1). We also observed changes in the inspiratory motor pattern consisting of transient reductions in PS EMG amplitude usually associated with increases in RR. In addition, alterations in BP, increases in ThAr activity, and depression of inspiratory PCA EMGs were observed. We did not detect any ABD or ThAr activity in the control period; thus the level of these EMGs in preinjection control period was considered to be zero, and their increases after the DLH microinjection were normalized to the maximum burst during cough. In seven cats injected with the higher dose of DLH, there were transient increases in BP (146 ± 8 from 129 ± 6 mm Hg; P < 0.01), RR (37 ± 6 from 30 ± 4 breaths/min; P < 0.05), ABD EMG activity [48 ± 15% on the contralateral side (P < 0.05) and 18 ± 5% on the ipsilateral side (P < 0.05)], and suppression of contralateral PS activity (41 ± 14% of preinjection control; P < 0.01). ABD discharge induced on the contralateral side was significantly higher than that on the ipsilateral side (P < 0.05). These peak changes typically were completed within 1 min, and beyond this period of time the parameters were not significantly different from control (Table 3). The expiratory ThAr EMG activity recruited by the DLH microinjection (amplitude 38 ± 25%) as well as inspiratory PCA EMG activity (86 ± 12% of preinjection control) was not statistically significant compared
with the control. In four cats injected with lower dose of DLH, the alterations of monitored cardiorespiratory parameters were qualitatively similar (and less pronounced) to those observed in the group of animals with the higher dose of DLH.

In seven aCSF-injected animals (8 locations) in some cases we observed slight (range 0–13%) and nonsignificant changes in the monitored cardiorespiratory parameters after the microinjection.

We histologically reconstructed (Fig. 3A) the position of five injection locations in animals that received the higher dose of DLH (Fig. 3B). All these injections were placed in the ventral and ventromedial caudal nucleus retroambigualis (cNRA) or in its ventromedial border (Fig. 3B). Seven of eight aCSF injected locations were reconstructed. They were found in the central, medial, and ventral (2 injections) part of cNRA, and three injections were localized at and close to the lateral edge of cNRA (Fig. 3B).

**DISCUSSION**

The major finding of this study is that the cough reflex was suppressed by excitation of neurons in the region of the caudal ventral respiratory column with microinjection of the EAA agonist DLH. This cough suppression manifested as a reduction in cough number as well as in abdominal EMG amplitudes lasted up to 15 min after the injection.

This is the first report to investigate the effects of local microinjection of an excitatory amino acid (EAA) agonist into the cVRC on cough. DLH is a nonspecific EAA agonist and was used in this study to excite neurons in the region of the micropipette tip. Our present experiments were based on excitation of EAA receptors by single injections of relatively small volumes (and doses) of DLH, which presumably affected a limited number of neurons in the cVRC of the cat. Our micro-injections were unlikely to have directly modified the activity of neurons at distance >0.5 mm away from the micropipette tip (32). The concentrations and doses of DLH used here also were unlikely to have caused significant long-lasting and extensive depolarization block (for a detailed discussion of mechanisms and limitation of the technique, see Refs. 28, 32). Consistent with this concept was our observation that we could reproduce DLH-induced cardiorespiratory responses within 10 min of a prior injection.

The microinjections made in these experiments were in or very near to the nucleus retroambigualis, a region that is associated with a high concentration of expiratory premotoneurons and is considered to be part of the ventral respiratory column (1, 20). The DLH microinjections excited expiratory premotor pathways because we observed increases in ABD motor discharge particularly on the contralateral side to the injection. In the cat, expiratory premotoneurons in cVRC have axons that cross the medullary midline before descending the spinal cord (1, 29). We also observed increases in ABD motor activity on the ipsilateral side, presumably because some of these descending axons cross at the segmental level and/or synapse on interneurons that have crossed axons (24, 30). These observations are consistent with those of Bongianni et al. (10), who employed DLH microinjections in cVRC to study the effects of excitation of neurons in this area on breathing in cats.

**Table 1. Effect of DLH microinjections in region of cVRC on laryngeal abductor and adductor activity during cough**

<table>
<thead>
<tr>
<th>PCA, % of control</th>
<th>ThAr, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inspiration</td>
<td>Expiration</td>
</tr>
</tbody>
</table>

Preinjection

0-5 min post-DLH

10-15 min post-DLH

Recovery

Values are means ± SE. Data are abdominal muscle amplitudes as a percentage of control. ThAr = thyroarytenoid. PCA = posterior cricoarytenoid. ThAr = thyroarytenoid. PCA = posterior cricoarytenoid. ThAr = thyroarytenoid.

**Table 2. Effect of DLH microinjections in region of cVRC column on cough phase durations**

<table>
<thead>
<tr>
<th>CTt</th>
<th>CTl</th>
<th>CTtot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preinjection</td>
<td>1.00±0.16 s</td>
<td>1.69±0.46 s</td>
</tr>
<tr>
<td>0-5 min post-DLH</td>
<td>1.06±0.14 s</td>
<td>1.86±0.33 s</td>
</tr>
<tr>
<td>10-15 min post-DLH</td>
<td>1.01±0.16 s</td>
<td>1.86±0.43 s</td>
</tr>
<tr>
<td>Recovery</td>
<td>1.05±0.17 s</td>
<td>1.97±0.66 s</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data are cough inspiratory (CTt), cough expiratory (CTl) phase and total cough cycle (CTtot) durations are expressed in seconds as well as the percentages of the control preinjection CTtot.
afferent input, by suppression of the excitatory gating mechanism, or by a direct action on the respiratory and cough pattern generator (Fig. 4). These effects will result in either prolongation of individual phases of coughing and/or a reduced ability of the pattern generator to reconfigure for the production of cough (e.g., inhibition of input from the gating mechanism). The fact that no significant alterations of phase durations (including the duration of cough ABD bursts) were detected was similar to the actions of centrally administered cough-suppressant drugs (6, 7). In addition, the relative proportions (and significant difference) of normalized CTI to CTE were suppressed drugs (6, 7). In addition, the relative proportions (including the duration of cough ABD bursts) were detected and suppressed even when tonic ABD discharge, and this depression was bilateral. Moreover, cough ABD amplitudes were suppressed even when tonic ABD activity due to the DLH injection was elevated. These observations are consistent with a loss or suppression of presynaptic drive to the expiratory premotoneurons specifically during cough and not with a generalized suppression of expiratory motor output.

Alternatively, although it is accepted that the reflex coughing is produced by brain stem neuronal network, we cannot rule out the existence of a spinal descending component of the cough suppression mechanism that could have been activated by microinjection of DLH in cVRC. Such a neuronal pathway could directly reduce cough-related ABD motor discharge and/or activate ascending tract neurons that could influence the brain stem cough mechanism.

Taken together, results from this study are consistent with the hypothesis that there is an endogenous cough-suppressive neuronal mechanism or network involving neurons located in the cVRC (Fig. 4). In addition to the observations that were already noted, the cough suppression lasted considerably longer (−15 min) than the transient perturbations of breathing and/or BP induced by DLH microinjection. This observation during cough were found in the medullary respiratory areas, particularly in cVRC (21, 41). Arita et al. (1) described some inspiratory cells intermingled among expiratory neurons in cVRC. These inspiratory units were not antidromically activated from the spinal cord or vagus nerve. This population of neurons, populations of silent neurons, or nonbreathing modulated neurons in or near the cVRC could be responsible for the DLH-induced cough suppression. Very little is known about possible axonal projections and/or connectivity of such groups of neurons. Anatomic studies report extensive projections from the region of cVRC to the multiple locations in the medulla and pons (44) despite the lack of such projections from spontaneously active expiratory bulboспinal neurons. It is possible that the results of the anatomic studies are related primarily to one or more populations of neurons that were activated by DLH in this study. Indeed, there is a well-defined neuronal pathway from periaqueductal gray to neuronal populations in cVRC. Silent neurons in the region of the cVRC can be antidromically activated from the contralateral ventral respiratory column rostral to the obex. These neuronal pathways are presumably involved in the expression of vocalization (42, 51) but could be involved in the expression of other behaviors such as cough.

Unilateral microinjection of DLH reduced cough-related ABD discharge, and this depression was bilateral. Moreover, cough ABD amplitudes were suppressed even when tonic ABD activity due to the DLH injection was elevated. These observations are consistent with a loss or suppression of presynaptic drive to the expiratory premotoneurons specifically during cough and not with a generalized suppression of expiratory motor output.

Table 3. Mean arterial blood pressure, heart rate, end-tidal CO2, respiratory rate, and duration of inspiratory and expiratory phases of breathing before and after the DLH microinjection

<table>
<thead>
<tr>
<th></th>
<th>Preinjection</th>
<th>1–5 min</th>
<th>Post-DLH</th>
<th>10–15 min</th>
<th>Post-DLH</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP, mmHg</td>
<td>129±6</td>
<td>135±7</td>
<td>133±7</td>
<td>129±6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR, breaths/min</td>
<td>224±8</td>
<td>225±7</td>
<td>227±8</td>
<td>222±6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETCO2, breaths/min</td>
<td>31±1</td>
<td>31±1</td>
<td>31±1</td>
<td>32±1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR, breaths/min</td>
<td>32±4</td>
<td>33±4</td>
<td>30±3</td>
<td>31±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ti, s</td>
<td>0.64±0.06</td>
<td>0.62±0.05</td>
<td>0.65±0.05</td>
<td>0.65±0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Te, s</td>
<td>1.46±0.20</td>
<td>1.37±0.19</td>
<td>1.44±0.17</td>
<td>1.47±0.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. BP, arterial blood pressure; HR, heart rate; ETCO2, end-tidal CO2; RR, respiratory rate; Ti, duration of inspiratory phase of breathing; Te, duration of expiratory phase of breathing.

Fig. 3. Reconstruction of DLH injection sites. A: example of a transverse medullary section with a DLH injection site (fluorescent beads, white spot in the middle) in the dorsal part of nucleus reticularis gigantocellularis (NRA). The shape of the labeled region was partially protracted from the dorsosentral direction consistent with the track of the micropipette. Injection locations were considered to be the point of maximal intensity of the marker in the most ventral part of the labeled area. B: summary of injection sites in the caudal medulla highlighting the position of NRA. In the right side of B, the enlarged area of NRA shows representative locations of 5 identified DLH microinjection (+) and 7 reconstructed artificial cerebrospinal fluid microinjection spots (●).
and suppression of inspiratory activity. These effects have been induced by ABD and ThAr discharges, a transient increase of BP, and the larynx is involved in this effect, other more bilaterally, whereas our study was restricted to single microinjections performed by Bongianni et al. (11) performed on rabbits. This suggests an excitatory influence of the cVRC cough-suppressive element on the gating mechanism. However, inhibition of other components of cough network cannot be ruled out.

indicating that the prolonged reduction is specific for cough and does not represent a generalized suppression of any respiratory motor output. While we believe that local excitation of neurons in the region of the cVRC is involved in this effect, other more complex mechanisms could play a role. For example, in the immediate region of the micropipette, depolarization block of spontaneously active neurons could occur. This mechanism may lead to disfacilitation of other neuron groups. Furthermore, the DLH microinjections could lead to suppression of local and/or distant neurons via the excitation of inhibitory interneurons.

A recent study of Bongianni et al. (11) performed on rabbits has shown that multiple microinjections of EAA antagonists into the area of cVRC eliminated both the inspiratory and expiratory components of mechanically induced cough. Suppression of coughing by EAA antagonists suggests an existence of an excitatory neuronal population with ionotropic EAA receptors in the region of cVRC involved in cough expression. Multiple injections performed by Bongianni et al. were placed to cover a large proportion of the rabbit cVRC bilaterally, whereas our study was restricted to single microinjections. It is unknown whether EAA antagonists would have similar actions in the cat.

PCA and ThAr activities did not change significantly when cough ABD amplitudes were reduced. Furthermore, the laryngeal motor pattern during cough can be variable in the same as well as in different experimental preparations (2, 16, 34, 37, 43). These observations support the concept that cranial and spinal motor outputs are subject to different regulation specifically during cough (36, 43).

The DLH microinjections into the area of cVRC in our study induced ABD and ThAr discharges, a transient increase of BP, and suppression of inspiratory activity. These effects have been described by others (10, 49). On the other hand, we did not observe long apneic responses as reported by Bongianni et al. (10). We typically observed suppression of PS inspiratory amplitudes and increased RR after the microinjection of DLH in contrast to their findings. These differences may be due to the lower doses of DLH in our study (maximum 1.5 nmol) compared with that (1.6–4.8 nmol) in the experiments of Bongianni et al. Moreover, the animals in their experiments were vagotomized and artificially ventilated, which is markedly different from our preparation.

The neuropharmacological mechanisms responsible for control of the putative cough-inhibitory population of neurons in the cVRC are currently unknown. These mechanisms are likely be important in understanding the functional relevance of our results in the regulation of cough. DLH is a nonspecific EAA agonist, and it was used solely as a tool for excitation of neurons in the cVRC. The use of this drug did not allow us to identify specific EAA receptor subtypes that were responsible for the effects that we observed. The role of inhibitory systems such as GABAergic and glycnergic receptor mechanisms may be very important in the regulation of the cough-suppressive mechanism. GABA_A receptors participate in gain modulation of cVRC expiratory premotoneurons (48), although the role of these receptors in controlling the activity of other neurons in the region is not well understood. Peripheral or central administration of the specific GABA_B receptor agonist, baclofen, inhibits cough in a dose-dependent manner (5). This cough suppressant action of baclofen was significantly reduced by pretreatment with a specific GABA_B receptor antagonist, but the antagonist alone had no effect on the expression of cough (4). These results suggest that GABA_B receptor mechanisms do not participate in a tonically active control system for cough expression. However, they may have a role in regulating the expression of cough under specific conditions that did not occur in our experiments. Although glycnergic synaptic mechanisms are important in various functions of the brain stem respiratory motor system (12), the role of glycine receptors in the control of neuron excitability in the region of the cVRC is not clear (50). Further work is necessary to more clearly define the neurochemical control of this cough control system.

Results from this study have prompted us to revise our laboratory’s functional model of the cough neurogenic system (7, 8). In addition to an excitatory regulatory element (the gate) that was proposed in earlier versions of the model, we now propose the presence of an inhibitory element (Fig. 4), which we term a “cough suppressor.” The exact conditions under which this cough-suppressive element may act are unknown. However, unlike the gating mechanism, we have identified a particular medullary region that contains at least some of the neural components that contribute to this cough suppressive element. The cough suppressor does share an important characteristic with the gate in that its influence on cough can be manifest in the absence of an action on breathing. In essence, the gate and cough suppressive element are not critical components of the breathing pattern generation system. These concepts are supportive of our laboratory’s recent hypothesis (9) that the neurogenic mechanisms for airway defensive behaviors (such as cough) are functionally organized in a holar chical system (25). That is, the expression of these behaviors is controlled by novel elements that impart unique regulation to
the system. For example, unlike breathing, cough in humans and animals is suppressed by hypoxia (13, 46). Furthermore, hypercapnia and hypocapnia have little effect on cough (33). According to this hypothesis, the breathing pattern generator is important in the production of cough (38–40) but is itself regulated by other control elements (9).

GRANTS
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