Respiratory and circulatory effects of parietal pleural afferent stimulation in rabbits

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—Respiratory symptoms accompanying pleural diseases combine dyspnea, tachypnea, rapid shallow breathing, and sometimes hypotension. There are no experimental data on the changes in respiratory and circulatory functions elicited by the activation of pleural afferents. After removal of all muscles covering the 5th to 10th intercostal spaces, we investigated in paralyzed, vagotomized rabbits the changes in phrenic discharge, transpulmonary pressure, and systemic arterial pressure in response to an outwardly directed force exerted on the parietal pleura or the local application of solutions containing lactic acid or inflammatory mediators. Mechanical stimulation of the pleura induced an immediate decrease in both integrated phrenic discharge and arterial blood pressure, the responses being positively correlated with the magnitude of force applied on the pleura. No accompanying changes in ventilatory timing, transpulmonary pressure, or heart rate were measured. Lactic acid solution also elicited an inhibition of phrenic activity and a fall in blood pressure. Section of the internal intercostal nerves supplying the stimulated intercostal spaces totally abolished the responses to mechanical stimulation or lactic acid. An inflammatory mixture elicited only modest respiratory and circulatory effects. We concluded that an acute mechanical distension of the parietal pleura as well as its chemical stimulation by lactic acid elicit a marked inhibition of phrenic motoneurons combined to a reduction of the sympathetic outflow to the circulatory system.

Pleural diseases cause pain, dyspnea, tachypnea, and rapid shallow breathing, and sometimes hypotension, which only occurs under specific circumstances, e.g., tension pneumothorax, overwhelming infection of the pleural space causing sepsis, and associated severe cardiac dysfunction (26, 38). The human study by Capps (6) showed that mechanical stimuli applied to the peripheral margin of the diaphragm of awake subjects elicited referred pain to the thorax, and the author speculated about the role of intercostal nerves in producing the sensations experienced by these patients. Besides, in the human as well as animal literature, we found no experimental data investigating cardiorespiratory reflex effects that may be attributable to activation of pleural afferents.

On the basis of our previous data of the behavior of pleural afferents in internal intercostal nerves (19), we conducted an experimental study in the same species to explore the consequences of mechanical or chemical stimuli applied to the parietal pleura on the breathing pattern (phrenic discharge) and the circulatory function (blood pressure, heart rate). Some data exist in the literature on the respiratory effects of internal intercostal nerve stimulation, but they are only based on electrical stimulation of the whole nerve trunk or mechanical activation of nerve endings, but not on their chemical activation.

MATERIALS AND METHODS

Animal Care and General Preparation

The animal experiments were performed in accordance with the requirements of the ethic committee of the Jean Roche Institute (School of Medicine, Marseille, France). Our laboratory has been granted a license from the French government to conduct animal research.

Seven adult rabbits (body weight = 2.5 to 3.0 kg) were used. They were anesthetized by an injection of ethyl carbamate, 1 g/kg (urethane) in the marginal ear vein. The external jugular vein was cannulated to continue anesthesia by subsequent injections and to perfuse the animals with saline containing adrenalin (0.3 μg·kg⁻¹·h⁻¹) to maintain the systolic blood pressure in the range 100–130 mmHg after thoracotomy. A heating pad maintained rectal temperature in the range 37–38°C. The animals were paralyzed at hourly intervals by intravenous injections of pancuronium bromide (Pavulon, Organon Technika, France, 0.4 mg/kg).

Throughout and after the operative procedure, the adequacy of the level of anesthesia was judged from the changes in blood pressure, heart rate, and pupil size evoked by noxious stimuli, the changes in these variables governing the injection of hourly doses of ethyl carbamate. At

IN A PREVIOUS STUDY IN RABBITS, we identified afferents in internal intercostal nerves that were activated by mechanical and/or chemical stimulation of the thoracic pleura (19). The nerve responses were attributed to the selective activation of pleural afferents because they existed after complete removal of all muscles covering the studied intercostal spaces. Because the conduction velocity of these afferents ranged between 0.5 and 14 m/s, we deduced that they belonged to the group III (thin myelinated) and IV (unmyelinated) fibers. Most units (97%) were activated by mechanical stimulation of the pleura (local positive pressure range = 4.5 to 8.5 cmH₂O), and we found a linear relationship between the discharge rate of afferents and the force applied to the thoracic wall. However, only 29% of this nerve population was purely mechanosensitive. The other units also responded to lactic acid and/or inflammatory mediators and were considered multimodal receptors. The response to lactic acid was roughly proportional to its concentration.

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Surgical Procedure

Because mechanical stimulation of the visceral pleura and/or the heart could sometimes occur when a piece of gauze was applied against the thoracic wall, both vagus nerves were cut at the cervical level to eliminate the major cardiopulmonary innervation.

A large median sternotomy was performed from the xyphoid to the manubrium sterni to bilaterally expose the entire thoracic pleural area. On both sides, the thoracic muscles covering the 5th to 10th intercostal spaces, such as the pectoralis major and minor, serratus anterior, triangularis sterni, and abdominal muscles (transversus and rectus abdominis and internal oblique) were dissected and removed. In these interspaces, we used an operating microscope (×40, OPM II Zeiss) to carefully remove the external and internal intercostal muscles and to leave the parietal pleura intact. The more proximal muscles attaching to the ribs (dorsal parts of the intercostals, ilio-costalis, or levator costae) were not removed, but all the muscles distal to the points of nerve sections performed at the end of the protocol were removed.

Physiological Measurements

A tracheotomy was performed, and the animals were ventilated at constant volume (10 ml/kg) and frequency (24–25 min−1) with a Harvard volumetric pump. O2 and CO2 fractions were respectively measured with rapid pyrolytic and infrared gas analyzers. End-tidal CO2 fraction was maintained between 0.03 and 0.04, and inspired O2 concentration was fixed at 0.30.

The tracheal pressure was measured with an electromanometer (Statham PM5) connected to a side arm of the tracheal cannula. Because the chest was largely opened and the animals were mechanically ventilated, tracheal pressure measurements reflect the variations of lung resistance. Lung compliance was maintained stable by performing frequent pulmonary inflations (3 × stroke volume of the pump) (25).

The left carotid artery was catheterized for measurements of blood pressure and heart rate, with an electromanometer (Statham P23 Dn), and also for blood gas analyses (Radiometer ABL 330, Copenhagen, Denmark).

A phrenic root was dissected free in the neck on each side. The right phrenic nerve was dissected free in the neck on each side. The right phrenic root was dissected free in the neck on each side. The left carotid artery was catheterized for measurements of blood pressure and heart rate, with an electromanometer (Statham P23 Dn), and also for blood gas analyses (Radiometer ABL 330, Copenhagen, Denmark). A phrenic root was dissected free in the neck on each side. The right phrenic nerve was dissected free in the neck on each side. The left carotid artery was catheterized for measurements of blood pressure and heart rate, with an electromanometer (Statham P23 Dn), and also for blood gas analyses (Radiometer ABL 330, Copenhagen, Denmark).

Response to lactic acid (7 rabbits). A gauze piece (10 × 20 mm) soaked in lactic acid solution (40 mM) was positioned for a 10-s period at the same thoracic level. No pressure was continuously exerted on the gauze piece. In five animals, we also compared the response to 20 mM (n = 7; pH = 5.10) and 40 mM (n = 11; pH = 4.7) lactic acid solutions. These lactic acid concentrations are often measured in pleurises (5).

Response to the application of a gauze piece soaked in an inflammatory mixture composed of bradykinin-5-HT-histamine-PGE2 at 10−5 M concentration (pH = 6.7) (6 rabbits, n = 9). The composition of this mixture was the same as in our previous study (19) and also that by Wedekind (36). The contact of the pleura with the inflammatory mixture solution was maintained for a 30-s period.

Lactic acid and inflammatory mixture tests were randomized among animals.

Chemical stimulation obliged us to transiently apply the gauze soaked in solutions against the thoracic wall and thus to elicit an initial mechanical stimulation. We preferred to use this mean and not to rinse the parietal pleura with chemical solutions to avoid any spreading of chemicals over the thorax. We also avoided spreading of chemicals over the internal thoracic wall by limiting the soaking of gauze in each solution. Between two successive applications of chemicals, the thoracic side was rinsed with a large amount of warmed saline. Fifteen minutes lapsed between two successive test agents. The chemical solutions in which the application gauze was soaked were maintained at 37°C in a thermostatic water bath.

Blank tests for chemical stimulation consisted of application of gauze soaked in warmed saline serum for the same duration. They were reproduced by touch stimuli eliciting the same mechanical stimulation.

A whole trial in one pleural side consisted of four to seven mechanical stimulations, which were followed by one blank chemical test and then by one or two applications of lactic acid solution (20 mM then 40 mM) and one application of the inflammatory mixture.

In two animals, mechanical stimulation was applied on the thoracic pleura when recording both the ipsi- and contralateral phrenic nerves. At the end of each experiment, the internal intercostal nerves in 5th to 10th spaces were dissected and freed from surrounding tissues as described by De Troyer and Legrand (11). The final section of internal intercostal nerves was distal to the branch point for the lateral nerve branch innervating external abdominal oblique muscle. Before section of intercostal nerves, one mechanical stimulation was repeated to assess the persistency of control phrenic and circulatory responses. After nerve sections, we repeated mechanical stimulations and application of the 40 mM lactic acid solution on the thoracic pleura and measured the response of the ipsilateral phrenic nerve and the changes in blood pressure and heart rate.
Statistical Analysis

The baseline values of transpulmonary pressure, systolic and diastolic blood pressure, heart rate, peak integrated phrenic discharge, and total breath duration were averaged for a 30-s period before application of each test agent and were expressed as means ± SE. Then, the significance of maximal changes in each variable induced by each test agent was determined with respect to the corresponding averaged baseline value. For phrenic nerve recording, the changes in amplitude and duration of the phrenic discharge were assessed once per cycle to determine their maximal variations. For the majority of mechanical and chemical tests, the depression of phrenic activity remained stable for a few breaths (5–12). Thus reported data corresponded to the averaged changes in amplitude and duration of phrenic discharge. We used an analysis of variance for repeated measures followed by Student-Newman-Keuls post hoc test to indicate the direction and magnitude of the variations between the different conditions. Data processing was conducted on absolute values by using a software program (SigmaStat, Jandel, Chicago, IL). Because there were no significant differences between the baseline values of physiological variables measured in each animal throughout a whole experiment, we expressed the changes in each variable elicited by pleural stimulation as a percentage of the corresponding prestimulation level. Regression analyses between force applied on the thoracic pleura and the changes in phrenic discharge and blood pressure were also performed.

RESULTS

Responses to Mechanical Stimulation of the Thoracic Pleura

As illustrated in Fig. 1, the response to mechanical stimulation resulted in an immediate decrease (1.2 ± 0.3 s) in peak amplitude of integrated phrenic discharge and a slightly longer latency (2.4 ± 0.5 s) for the first drop in arterial pressure, the circulatory response often adapting throughout the period of mechanical stimulation. The decrease in amplitude of phrenic discharge and also systolic and diastolic blood pressures was positively correlated with the magnitude of force (Fig. 2). We found no correlation between the stimulus duration and the magnitude of either phrenic or blood pressure response. Both baseline spontaneous respiratory frequency (36 ± 8 min⁻¹), counted from integrated phrenic nerve recording, and phrenic...
discharge duration (0.61 ± 0.08 s) did not significantly vary in response to mechanical stimulation, even when the stimulus was maximal. No significant variation of transpulmonary pressure was measured, and HR (baseline: 260 ± 22 min⁻¹) did not vary at all.

The phrenic response seems to be strictly unilateral because no changes in phrenic discharge were measured when the contralateral phrenic nerve was recorded during mechanical stimulation of the thoracic pleura. Figure 3 gives an example of seven trials conducted in two animals.

In all animals, section of the internal intercostal nerves supplying the stimulated intercostal spaces abolished both the phrenic and blood pressure responses (Fig. 4).

Responses to Chemical Stimulation

Lactic acid. Both the 20 and 40 mM solutions elicited significant phrenic and blood pressure changes (Fig. 5). In the seven rabbits, application of gauze soaked in lactic acid early
elicited phrenic inhibition and blood pressure decrease (2.3 ± 1.2 and 3.8 ± 1.0 s, respectively), which may be attributed to the initial modest mechanical stimulation. Then, opposing the phrenic response to mechanical stimulation that disappeared after removal of pressure applied to the chest wall (Figs. 1, 3, and 4), the respiratory and also the circulatory effects elicited by lactic acid continued to develop despite the absence of persistent changes in force applied against the thoracic wall (Fig. 5). The response was significantly greater for trials performed with the greater lactic acid concentration (Table 1). As for mechanical stimulation, we did not measure any significant variation of spontaneous respiratory frequency, transpulmonary pressure, and HR. In all cases, the blood pressure fall lasted more than the phrenic inhibition and, as shown in Table 1, the fall in diastolic blood pressure was significantly higher than that of systolic pressure. Final section of the internal intercostal nerves supplying the stimulated intercostal spaces totally abolished both the respiratory and circulatory responses to lactic acid.

**Inflammatory mixture.** The inflammatory mixture elicited a modest but significant decrease in both phrenic discharge (−15 ± 4%; *P* < 0.05) and blood pressure (mean decrease in systolic pressure was −13 ± 2%; and mean decrease in diastolic pressure equaled −10 ± 2%, *P* < 0.05). The phrenic and circulatory response latencies were 5.3 ± 1.4 and 7.8 ± 2.0 s, respectively.

Unlike the durable responses to lactic acid or inflammatory mixture, application of gauze soaked in warmed physiological saline serum (blank chemical test) only induced transient inhibitory effects limited to the duration of mechanical stimulation.

**DISCUSSION**

The present study reports inhibitory effects of mechanical and chemical stimulation of the thoracic parietal pleura on both the phrenic nerve discharge and systemic blood pressure, with no associated changes in the ventilatory timing and heart rate. Both the changes in phrenic discharge and the circulatory response were proportional to the magnitude of mechanical stimulation and also depended on the concentration of lactic acid solution applied against the parietal pleura. Application of a mixture of inflammatory mediators also elicited an inhibition of the phrenic nerve discharge and a blood pressure decrease. The response was reflex because the section of internal intercostal nerves totally suppressed both the respiratory and circulatory effects. We supposed that muscle afferents cannot be involved in the reflex phrenic and circulatory responses elicited by pleural stimulation. Indeed, we ensured the destruction of

Table 1. *Maximal respiratory and blood pressure changes in response to local application of 20 mM or 40 mM lactic acid solution on the thoracic pleura*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔEphr, %</th>
<th>ΔPa systolic, %</th>
<th>ΔPa diastolic, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA 20 mM</td>
<td>−29±5*</td>
<td>−19±3*</td>
<td>−23±4*</td>
</tr>
<tr>
<td>LA 40 mM</td>
<td>−36±6</td>
<td>−25±2</td>
<td>−31±3‡</td>
</tr>
</tbody>
</table>

Values are means ± SE, given as percent of baseline. Ephr, phrenic activity; Pa, blood pressure; LA, lactic acid; Δ, change. *Significant difference (*P* < 0.05) between the effects of 20 mM and 40 mM solutions. Significant differences between systolic and diastolic blood pressure variations: † *P* < 0.05; ‡ *P* < 0.01.
all thoracic and abdominal muscles covering the stimulated and also the adjacent interspaces and also of muscles inserted on the ribs delimiting the studied pleural area. Moreover, intercostal nerves contain a large proportion of fast conducting fibers connected with muscle spindles and Golgi tendon organs (33), and solely slow-conducting fibers innervated the parietal pleura (19).

In our protocol, the mechanical distension of the thoracic pleura also distended the thoracic wall, and thus could activate the costovertebral joints receptors described by Godwin-Austen (14). These slowly adapting receptors are localized in the capsule of the costo-transverse joint and signal the rib joint position and the direction and velocity of movement. Recording the electromyographic activity in external intercostal inspiratory muscles, De Troyer (10) reported an increased activity when the normal cranial motion of the ribs was reduced by external force and a decreased inspiratory muscle activity when the cranial motion of the ribs was augmented. Shannon (32) measured a marked inhibition of the phrenic nerve activity during costovertebral joint movements. In our study, the pressure applied to the internal side of the thoracic wall indifferently induced cranial or distal motion of the ribs and thus could elicit either inhibitory or facilitating inspiratory reflexes mediated through the activation of costovertebral joint receptors. However, an inhibition of inspiratory activity was always noted. Another argument against possible reflex responses elicited by the activation of costovertebral joint receptors in our study was that the inhibitory phrenic response was abolished after section of internal intercostal nerves, distal to the branch point for their lateral branches and thus to the nerves innervating the costovertebral joint receptors recorded in dorsal-root filaments after laminectomy by Godwin-Austen (14). Moreover, we already showed that no fast-conducting afferents supplied the innervation of parietal pleura (19) and joint receptors are only connected with fast-conducting fibers. It seems also unlikely that mechanoreceptors in the costovertebral joints may be activated by the application of chemicals (lactic acid, inflammatory mediators, capsaicin) on the thoracic pleura.

In the present study, the phrenic inhibitory effects exerted by mechanical or chemical (lactic acid) activation of pleural afferents were limited to the side ipsilateral to the thoracic pleura, suggesting that their supraspinal projections were not involved. Indeed, first we measured no change in phrenic timing in response to either the mechanical or chemical stimulation, and second the inhibitory effect on the phrenic motor discharge strictly remained ipsilateral. We found no data on the effects of thoracic afferents stimulation on both the ipsi- and contralateral phrenic motoneurons. The mechanical stimulation of chest wall muscle seems to exert only excitatory influences on intercostal and phrenic motoneurons (22, 24, 30, 31, 33), opposing the inhibitory action exerted by thoracic pleural afferents on phrenic motoneurons here reported. We found one study in vagotomized dogs and cats that reported phrenic nerve inhibition in response to rapid compression of the chest with a pneumatic cuff (28): a possible mechanical activation of thoracic pleural afferents, not discussed in that study, cannot be discarded. Also Aminoff and Sears (3) reported an inhibition of ipsilateral inspiratory motoneurons in response to electrical stimulation of the central end of internal intercostal nerve that activated all afferents including those innervating the pleura.

We were surprised by the absence of changes in phrenic timing in response to pleural afferents activation, suggesting their absence of connections with the respiratory centers. Previous studies on the effects of the mechanical activation of intercostal mechanoceptors and costovertebral joint receptors reported consistent changes in respiratory frequency resulting from the supraspinal projections of these chest wall afferents on brain stem respiratory neurons via the cerebellum (35) and the Bötzing complex (33) and also directly to the dorsal and ventral respiratory groups (33). The existence of direct connections between parietal pleural afferents in internal intercostal nerves and phrenic motoneurons is similar to an inhibitory visceromotor reflex similar to that reported between the splanchnic afferents and the diaphragm (1, 2, 12). As for the pleural-to-phrenic reflex here described, splanchnic-to-phrenic reflex involves the activation of nonmyelinated afferent fibers (2) directly connected with ipsilateral phrenic motoneurons because the reflex persists in animals spinalized between C1 and C2 (1, 12). The splanchnic-to-phrenic reflex may explain inhibition of diaphragm electromyogram after peritoneal effusion with laparotomy (4) or simple laparoscopy (34). Thus there are numerous analogies between visceromotor reflexes elicited by mechanical activation of the pleura or peritoneum.

In our study, the chemical activation of pleural afferents also exerted both respiratory and circulatory responses that developed after a transient mechanical stimulation associated with gauze application. The magnitude of respiratory response was higher with the lactic acid solution than with the inflammatory mixture. Because the pH of lactic acid solutions was rather low, our lactic acid tests only mimicked the concentration of this substance measured in the majority of pleural effusions in which the lactic acid concentration was in the same range (5). However, the pleural fluid pH was markedly higher in pleurisy, because of the presence of buffers in the pleural fluids components (7). Our experimental animal study was very different than clinically relevant intervention, but it must be underlined that numerous other experimental studies on chemosensitive muscle afferents, including the diaphragmatic ones, are based on intra-arterial injections of the same low-pH lactic acid solutions (9, 17, 21). The buffers in blood and interstitial fluid must rapidly and markedly increase the pH of injected solution. We also noted that the phrenic and circulatory responses to inflammatory mediators was markedly less than those to lactic acid. This observation is not consistent with our previous electrophysiological data in the same species (19), where we measured a comparable activation of the pleural afferents in response to 40 mM lactic acid (+485%) and the same concentrations of bradykinin, 5-HT, histamine, and PGE2 (+639%). However, among the recorded pleural afferents in our previous study, the proportion of purely chemosensitive units was high (78%) when we tested the effects of lactic acid, but only 14% in response to the inflammatory mixture. On the whole, the chemosensitivity to lactic acid of the parietal pleura seems to prevail. This is not surprising because the bacterial synthesis of lactic acid is present in most of pleural effusions (27, 29), and its synthesis may also originate from the pleural inflammatory mesothelial cells (16, 23). Thus the present observations of modest respiratory effects elicited by pleural inflammation compared with the marked inhibitory action of mechanical pleural stimulation agree with clinical observations that mostly associate pleuritic symptoms to mechanical stimulation of the
pleural cavity. Indeed, the clinical review of pleuritic symptoms by Yernault (38) stated that the “pleuritic pain and dyspnoea are definitely intensified by deep breathing, coughing, laughing, or sneezing and relieved by anything that assists in immobilizing the affected side.”

There is no doubt as to the existence of specific inhibitory effects of pleural afferent activation on circulatory control in humans. In their review of the clinical literature, Noppen and Schramel (26) report that in primary spontaneous pneumothorax 30% of patients present with hypotensive faintness. In our animal study, hypotension, with parallel decrease in both systolic and diastolic arterial pressure, accompanied phrenic inhibition in response to mechanical pleural stimulation. During a sustained mechanical stimulation of the thoracic parietal pleura, it is worth emphasizing that blood pressure inconstantly varied in parallel with the development of phrenic inhibition. As illustrated in the figures, the blood pressure decrease either adapted while the phrenic inhibition progressed (Fig. 1) or the time courses of both variables were similar (Figs. 3–5). By contrast, lactic acid stimulation of pleural afferents elicited a durable and marked decrease in blood pressure, and in this case the fall in diastolic blood pressure was significantly higher than that of systolic pressure. We can totally discard the possibility that the blood pressure decrease accompanying the phrenic inhibition during application of gauze on the internal thora
cic wall could result from an abrupt fall in venous return because of cardiac and/or diaphragm compression. Indeed, first, the chest was largely opened and the rabbits were paralyzed, and second, the hypotension persisted after removal of the outwardly directed force in the case of lactic acid stimulation. Because the carotid sinus baroreflex was left intact, hypotension and the phrenic response may be also linked via a reflex mechanism. However, previous studies have clearly demonstrated that hypotension elicited hyper- and not hypoventilation (15, 18) and thus the phrenic inhibition here reported cannot result from the blood pressure fall that always followed the respiratory response. The activation of visceral afferents from the lungs also elicits hypotension. Thus injecting small amounts of bradykinin (20) or histamine (37) in the bronchial or pulmonary circulation to activate pulmonary vagal chemoreflexes as well as pulmonary hyperinflation to stimulate vagal mechanoreceptors in the airways (8) produce marked hypotension. It must be pointed out that vagotomy may have also suppressed any possible bronchomotor effects of the pleural afferent activation, but this intervention was necessary to discard any artifact due to the stimulation of pulmonary and also cardiac vagal afferents.

The present study clearly shows that mechanical or chemical stimulation of parietal pleural afferents elicits both respiratory and circulatory effects. However, we were only able to mimic positive pressure changes in the pleural cavity, a situation that occurs in rare circumstances such as cough, forced expiration, pneumothorax, pleurisy, and surgical pleuroscopy. Thus there is a major difference between our experimental protocol and the natural conditions in which pleural nerve endings are normally exposed to a negative (subatmospheric) pressure. We cannot conclude, therefore, that pleural afferents are activated during spontaneous breathing movements and that they would be able to modulate phrenic activity and sympathetic neural drive to arterial blood vessels.

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