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Thoracic Gas Volume Measurements in Paralyzed Mice

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Abstract—We have previously measured thoracic gas volume (V_{TG}) in spontaneously breathing mice using a whole body plethysmograph and have now extended our technique to allow for V_{TG} measurements during paralysis. BALB/c mice were anesthetized and placed in a body-box and ventilated *via* a tracheostomy cannula through the box wall. Box pressure (P_b) and tracheal pressure (P_{ao}) were measured during spontaneous breathing, and again after paralysis while mechanically compressing the chest. V_{TG} was much larger after paralysis (0.49 ± 0.06 ml, positive end-expiratory pressure $= 2$ cmH_2O) when compared with spontaneous breathing (0.31 ± 0.01 ml). External chest compression produced looping in the plots of P_b versus P_{ao} that was attributable to gradual changes in P_b upon release of the mechanical chest compression and had the character of thermal transients. Under the assumption that the rate of heating of the air in the chamber was proportional to the pressure applied to the animal's chest, and that any increase in air temperature was dissipated by heat absorption by the chamber walls, we developed an algorithm that corrected for the thermal events. This yielded similar results for V_{TG} (0.30 ± 0.02 ml) as obtained during spontaneous efforts. Our method may prove particularly useful when paralysis is required for the precise measurement of lung mechanics.

Keywords—Plethysmography, Boyle's law, Lung mechanics.

INTRODUCTION

Thoracic Gas Volume (V_{TG}) is measured routinely in lung physiology clinics using whole body plethysmography[5] and is important in the assessment of respiratory diseases. V_{TG} is also a major determinant of bronchial responsiveness.[2,4] The measurement of V_{TG} in mice is of particular interest because of the current importance of this species for respiratory research. Nevertheless, there are only a few reports in the literature of V_{TG} being measured in mice[7,12,14,15] and only one (from our laboratory) using plethysmography.[11] The plethysmographic technique requires that the mouse be able to make spontaneous breathing efforts against a closed airway in order to compress the thoracic gas and allow V_{TG} to be calculated on the basis of Boyle's law, mirroring the procedure used for human measurements.[10,11] However, spontaneous breathing can interfere with the precise measurement of lung function in mice, often requiring the animals to be paralyzed.[6] In this report, we describe a novel plethysmographic method for measuring V_{TG} in paralyzed mice in which the compressive action of the respiratory musculature is replaced by external chest compression.

METHODS

We studied BALB/c female mice (17–20 g, $n = 8$) obtained from Jackson Labs (Bar Harbor, ME). The animals were kept in our animal facility with free access to food and water and were acclimatized for at least 1 week before the experiments. A sentinel animal program ensured that the animals were free of common pathogens for this species. The experiments were approved by the Institutional Animal Care and Use Committee of the University of Vermont. The mice were anesthetized with intraperitoneal sodium pentobarbital (90 mg/kg), and the trachea was dissected free of surrounding tissue and cannulated with an 18-gauge cannula. The mice were then installed in a custom-designed chamber (Fig. 1) and the tracheal cannula connected to a port through the front face of the chamber. The chamber was determine to be leak free as tested before each experiment by pressurizing the chamber and observing an attached manometer.

A horizontal support fixed to the removable front face of the chamber allowed the animal to be conveniently prepared and installed prior to sealing the chamber. A rotating shaft was sealed with silicone-greased double O-rings through the rear face of the chamber. Rotating the shaft from outside the chamber caused a paddle connected to the inside end of the shaft to compress the chest of the mouse while it was inside the chamber. Chamber pressure (P_b) was measured with a piezoresistive differential pressure transducer (SC-24, SCIREQ Inc., Montreal, PQ, Canada) referenced to a second chamber with a long-time constant

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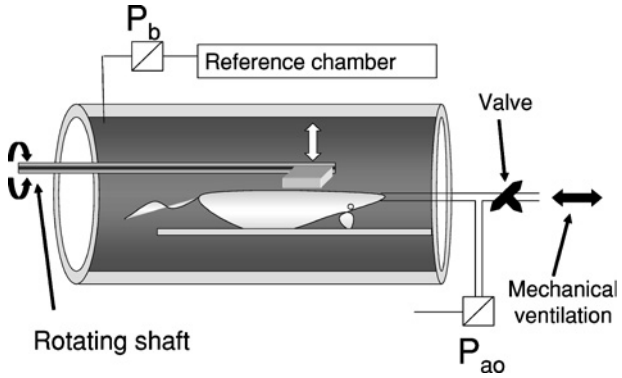

FIGURE 1. Plethysmographic chamber used to measure V_{TG}, *Zrs*, and *PV* curves. The chamber was essentially leak free (time constant >500 s). P_b was measured *via* a differential pressure transducer that was referenced to a second chamber with a volume of 1 l and a slow leak (time constant ~6 s) to atmosphere. P_{ao} was measured *via* another transducer at the tracheal opening. A movable shaft, with a paddle at the end, passing through the rear end of the chamber allowed the chest of the mouse to be compressed, thereby creating changes in P_b and P_{ao} as required calculating V_{TG}. The valve mounted between the ventilator and the trachea was closed during measurements.

(>6 s) leak to atmosphere. Airway opening pressure (P_{ao}) was measured with another piezoresistive gauge pressure transducer. The entrance to the externalized tracheal cannula was controlled by a stopcock that could either open the inspiratory line to a mechanical ventilator or close it for the measurement of V_{TG}. We established that rotation of the paddle inside an empty chamber did not affect P_b, confirming that rotation did not change the amount of shaft material inside the chamber or distort the chamber walls.

Mechanical ventilation was administered with a computer-controlled small animal ventilator (*flexiVent*, SCIREQ Inc. Montreal, PQ, Canada). Different levels of positive end-expiratory pressure (PEEP) were achieved by connecting the exhalation port of the *flexiVent* to a water trap. The *flexiVent* also allowed the measurement of the mechanical impedance of the respiratory system through the application of controlled perturbations in lung volume.[6,9,13,16]

When the inspiratory line was completely closed the animal attempted to breath against a closed airway. P_b then reflected decompression of the thoracic gas. Comparing P_b to P_{ao} gave a measure of the V_{TG} as determined by Boyle's law (see below). After paralysis, thoracic gas compression was achieved by compressing the chest with the paddle as described above.

Experimental Protocol

Mice were mechanically ventilated at 200 breaths/min with a tidal volume of 0.20 ml at each of three different levels of PEEP (0, 2, and 4 cmH_2O) applied in random order. Following 3 min of ventilation at each PEEP level, the animals were allowed to passively exhale against PEEP for 1 s after which a 2-s oscillation volume signal was applied to the lungs for the measurement of respiratory input impedance (*Zrs*) as described below. Following another minute of regular ventilation, a quasi-static pressure–volume (*PV*) curve was obtained as described below. Next, mechanical ventilation was suspended at the end of expiration and the airway was occluded for 10–15 s while the animals made spontaneous inspiratory efforts. This time of occlusion was necessary because the animals took some time to begin making breathing efforts after ventilation was interrupted. P_{ao} and P_b were recorded during this period and stored for later calculation of V_{TG}. After a further period of 1 min of mechanical ventilation, the airway was reoccluded for a second 10s period while the chest was manually compressed with the paddle at a rate of about 3 s^{-1}. During the initial 4–6 s following mechanical ventilation the mice generally stayed apneic, allowing us to obtain chest compression data before spontaneous breathing efforts started. Finally, the animals were paralyzed with intraperitoneal pancuronium (0.8 μg/kg) and ventilated for about 10 min after which *Zrs* and *PV* curves were again measured and the airway occlusion with chest compressions was repeated. All recorded signals were low-pass filtered at 30 Hz prior to being sampled at 256 Hz.

In a separate group of mice ($n = 5$) we measured V_{TG} by applying Archimedes' principle to open-chest animals ventilated for 1 min at a PEEP of 3 cmH_2O. At the end of the ventilation period the mice were euthanized with an overdose of intraperitoneal sodium pentobarbital, the trachea was tied off at end-exhalation and the lungs and heart excised *in toto*. The excised organs were then attached to the bottom of a volume-calibrated test tube and submerged in a known volume of saline which allowed the total volume (tissue plus air) to be determined. The organs were next weighed in air to give an estimate of the volume of tissue, assuming a tissue density of 1.04 gm/ml. The difference between the two volume estimates was taken as a measure of V_{TG}.

Data Analysis

Calculation of V_{TG}

We calculated V_{TG} from the changes in P_b and P_{ao} measured during either the closed airway breathing or external chest compressions on the basis of Boyle's law. We determined the ratio of changes in P_b to P_{ao} as the slope of the regression line between the two signals measured during the active phase of each spontaneous breathing effort. The relationship between P_b and P_{ao} during this phase was linear and displayed almost no hysteresis (Fig. 2). We determined the gas elastance (E_g) of the air in the chamber V_b in a separate experiment by applying known volume oscillations into the chamber at a rate similar to the mouse breathing frequency, with an animal and all other equipment *in situ*,

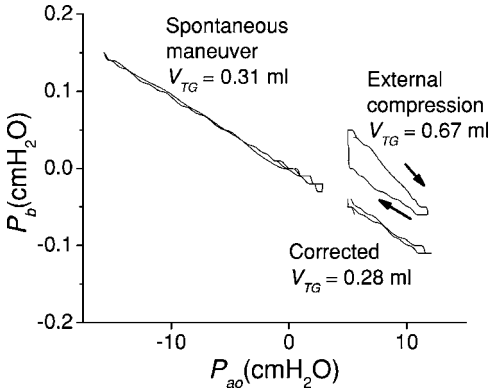

FIGURE 2. Representative plots of P_b versus P_{ao} obtained with the airway occluded during spontaneous breathing efforts, with external compression and when the chest compression data were corrected for thermal artifacts using Eq. 7. The looping of the external chest compression proceed in a clockwise direction as indicated by the arrows.

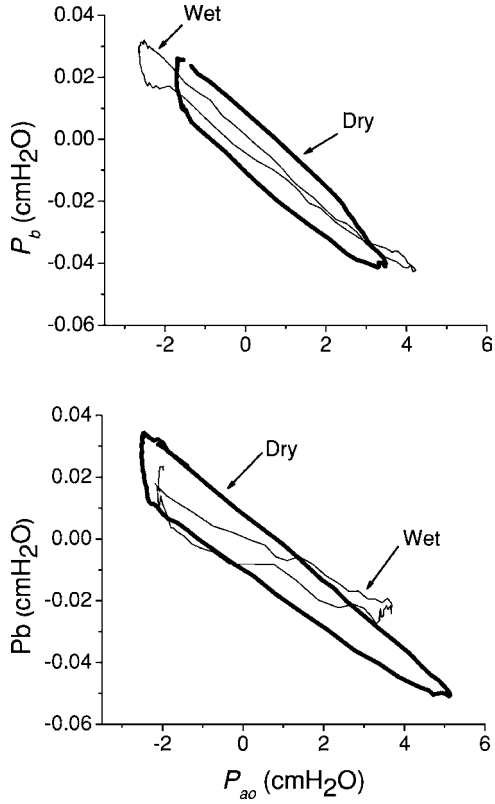

FIGURE 3. Representative plots of P_b versus P_{ao} from two mice obtained with the airway occluded during external chest compression. Thick lines represent data obtained in a dry mouse (Dry) and the thin lines represent data obtained in the same mouse with the fur soaked in water at body temperature (Wet).

while the corresponding oscillations in P_b were recorded. The ratio of changes in P_b to the amplitude of the applied oscillations gave an E_g of 11.1 cmH$_2$O·ml^{-1} (corresponding to a gas volume of approximately 90 ml). V_{TG} was calculated as

$$V_{TG} = \frac{\Delta P_b}{\Delta P_{ao}} \times \frac{1}{E_g} \times 1000 - S_d \qquad (1)$$

where S_d is the dead-space volume of the tubing connecting the pressure transducer to the trachea, and the factor 1000 is used to provide V_{TG} in units of ml.

In contrast to the measurements made during spontaneous breathing efforts, when external chest compression was employed we frequently observed marked looping in the plots of P_b versus P_{ao} (Fig. 2). The looping was attributable mostly to gradual changes in P_b seen upon release of each compressive stroke of the paddle. These changes in P_b had the character of thermal transients and they did not occur when we applied the technique to small volumes of gas enclosed in the sealed finger of a latex glove in place of the mouse. These thermal artifacts might be generated by trapped warm air within the fur of the mice. To confirm this, we conducted a separate experiment where we used two mice, which underwent the same chest compression protocol as described above, except at the end of the protocol their fur was soaked in water at body temperature and chest compressions were delivered again. The looping of P_b versus P_{ao} was reduced by wetting the fur (Fig. 3). We therefore concluded that these transients reflected the dissipation of heat released from the animal's body (e.g. trapped in its fur) each time it was compressed, and developed the following theory to correct for it. We made the assumption that the rate of heating of the air in the chamber was proportional to the pressure applied to the animal by the paddle, i.e. P_{ao}, and that any increase in air temperature was dissipated by heat absorption by the chamber walls which acted as a heat sink and remained at a fixed (ambient) temperature.

Let T_b be the temperature of the gas in the chamber relative to that of the chamber walls, and assume that any heat injected into the gas is absorbed by the walls at a rate proportional to the temperature difference between the gas and the walls (i.e. the heat is absorbed as a first-order process). This gives

$$\frac{dT_b(t)}{dt} = \alpha P_{ao}(t) - kT_b(t) \qquad (2)$$

where α and k are constants. Now, P_b is determined both by the degree of volumetric compression of air in the chamber and by T_b. These factors are approximately additive if T_b is small, thus

$$P_b(t) = -\gamma P_{ao}(t) + qT_b(t) \qquad (3)$$

where γ and q are two more constants. Combining Eqs. (2) and (3) gives

$$\frac{dP_b(t)}{dt} = -\gamma \frac{dP_{ao}(t)}{dt} + q\alpha P_{ao}(t) - qkT_b(t) \qquad (4)$$

We still have to eliminate T_b from Eq. 4. We see from Eq. 2 that T_b satisfies a first-order linear differential equation driven by P_{ao}. This means that T_b is given by the convolution of P_{ao} with an impulse response function that is a decaying exponential beginning at $t = 0$ with time-constant τ. Equation 4 can thus be written

$$\frac{dP_b(t)}{dt} = -\gamma \frac{dP_{ao}(t)}{dt} + q\alpha P_{ao}(t) - qkP_{ao}(t) \otimes \mathrm{H}(t)\, e^{-t/\tau} \tag{5}$$

where $\mathrm{H}(t)$ is the Heaviside step-function and $\otimes$ denotes the operation of convolution. Integrating Eq. 5 gives

$$\begin{aligned} P_b(t) = -\gamma p_{ao}(t) + q\alpha \int_0^t P_{ao}(t)dt - \tau qkP_{ao}(t) \otimes \mathrm{H}(t) \\ \times [1 - e^{-t/\tau}] + M = -\gamma P_{ao}(t) + q(\alpha - \tau k) \\ \times \int_0^t P_{ao}(t)dt + \tau qkP_{ao}(t) \otimes \mathrm{H}(t)\, e^{-t/\tau} + M \end{aligned} \tag{6}$$

where M is the constant of integration. In arriving at the second line of Eq. (6), we note that convolution with $\mathrm{H}(t)$ is equivalent to integration with respect to t from $t = 0$, and that the integral of the convolution of two functions equals the convolution of one of the functions with the integral of the other. As the parameters α, β, γ, τ, and q all are constants we can write Eq. 6 as

$$\begin{aligned} P_b(t) = AP_{ao}(t) + B \int_0^t P_{ao}(t)dt \\ + C[P_{ao}(t) \otimes \mathrm{H}(t)\, e^{-t/\tau}] + M \end{aligned} \tag{7}$$

We fitted the experimental data to Eq. 7 by systematically searching over a range of values of τ from 0 to 18 s, evaluating $A, B, C,$ and M at each step by multiple linear regression, in order to locate the set of parameter values that minimized the mean-squared difference between $P_b(t)$ and the right-hand side of Eq. 7. The second and third terms in Eq. 7 were evaluated by numerical integration and convolution, respectively. The best fit value of A obtained in this way was then taken as the corrected slope between P_b and P_{ao} for use in the calculation of V_{TG} (Eq. 1).

Calculation of Respiratory Mechanics

To measure the *Zrs*, we applied a 2 s broad-band volume perturbation signal to the lungs with the *flexiVent*. The volume signal consisted of the superposition of 13 sine waves having frequencies spaced roughly evenly over the range 1–20.5 Hz. *Zrs* was calculated from the displacement of the ventilator piston and the pressure in the ventilator cylinder as described previously.[6,9] Correction for gas compressibility as well as resistive and accelerative losses in the *flexiVent*, connecting tubing and the tracheal cannula were performed as described previously[16] using dynamic calibration data obtained by applying volume perturbations through the tubing and tracheal cannula first when it was completely closed and then when it was open to the atmosphere.

We interpreted the measurement of *Zrs* in terms of the constant phase model[8]

$$Zrs(f) = Raw + i2\pi f Iaw + \frac{G - iH}{(2\pi f)^{\alpha}} \tag{8}$$

where *Raw* is a frequency independent Newtonian resistance reflecting that of the conducting airways, *Iaw* is airway gas inertance, G characterizes tissue damping, H characterizes tissue stiffness, i is the imaginary unit, α links G and H, and f is frequency.[13]

Analysis of PV Curves

Quasi-static *PV* curves were obtained, starting at functional residual capacity as defined by each level of PEEP, by inflating the lungs in four 0.1-ml steps and then deflating them again in the same four steps, pausing at each step for 1 s.[17] Plateau pressure at each step was recorded and related to the total volume delivered. The range of the *PV* curve was chosen to encompass the range of V_{TG} we expected to see and thus allow for calculation of elastance over this range. Average elastance (E) was obtained by calculating the mean slope of the entire curve.

Statistics

Statistical differences between V_{TG} measured with spontaneous efforts and with external chest compression at different PEEP levels were established using two-way ANOVA. Statistical difference between E before and after paralysis calculated from the *PV* curves as well as *Raw*, *G*, and *H* were calculated using one-way ANOVA. A p value less than 0.05 was accepted as statistically significant.

RESULTS

Figure 4 shows V_{TG} obtained at different PEEP levels both with spontaneous breathing efforts and with external chest compression. V_{TG} was significantly smaller at all PEEP levels when measured with spontaneous breathing efforts than with chest compression, regardless of whether or not the animals were paralyzed ($p < 0.001$). However, V_{TG} obtained with chest compression increased with PEEP in parallel with the increases obtained with spontaneous breathing efforts. The looping observed in plots of P_b versus P_{ao} did not change appreciably with PEEP. When the thermal correction model was applied (Eq. 7) the V_{TG} values obtained with chest compression were comparable to those obtained with spontaneous efforts. V_{TG} measured according to Archimedes' principle gave a gas volume of

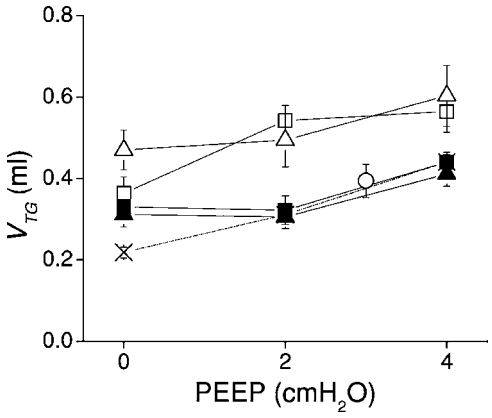

FIGURE 4. V_{TG} in anesthetized mice ($n = 8$) obtained from spontaneous breathing efforts (X, dashed line), from external chest compression prior to paralysis (squares) and from external chest compression after paralysis (triangles). Open symbols are data before correction for thermal artifacts; closed symbols are data after correction. Open circle is V_{TG} measured by water displacement (Archimedes' principle, $n = 5$). Error bars indicate SEM.

0.39 ± 0.04 ml at a PEEP of 3 cmH$_2$O which compares well to the values obtained with spontaneous efforts and with chest compression following thermal correction (Fig. 4).

Figure 5 shows *Raw*, *H*, and *G* obtained before and after paralysis. All three parameters decreased significantly ($p < 0.05$) with increasing levels of PEEP, but were not significantly affected by paralysis ($p > 0.05$).

Figure 6 shows the quasi-static *PV* curves obtained before and after paralysis of the mice. Table 1 shows that *E* obtained from the *PV* curves before and after paralysis were not significantly different at any PEEP level.

DISCUSSION

The current study is focused on the development and validation of a plethysmographic technique to measure V_{TG} in anesthetized, paralyzed mice. Whole body plethysmography has been the gold standard for lung volume measurements in the clinical setting since its invention in the 1950s[5] but has not been developed for small laboratory animals. The difference between whole body plethysmography and other means of obtaining lung volumes is that it measures the total volume of gas in the thorax, whether in free communication with the airways or not.[3] The crux of the plethysmographic method is to cyclically compress thoracic gas so that its reduction in volume can be related to its increase in pressure through Boyle's law. We have previously established[10,11] that reliable and reproducible measurements of V_{TG} can be obtained in mice using the conventional approach of having an animal make spontaneous breathing efforts against a closed airway. However, in settings where precise lung mechanics measurements (e.g. *Zrs* and *PV* curves) are required it is often desirable

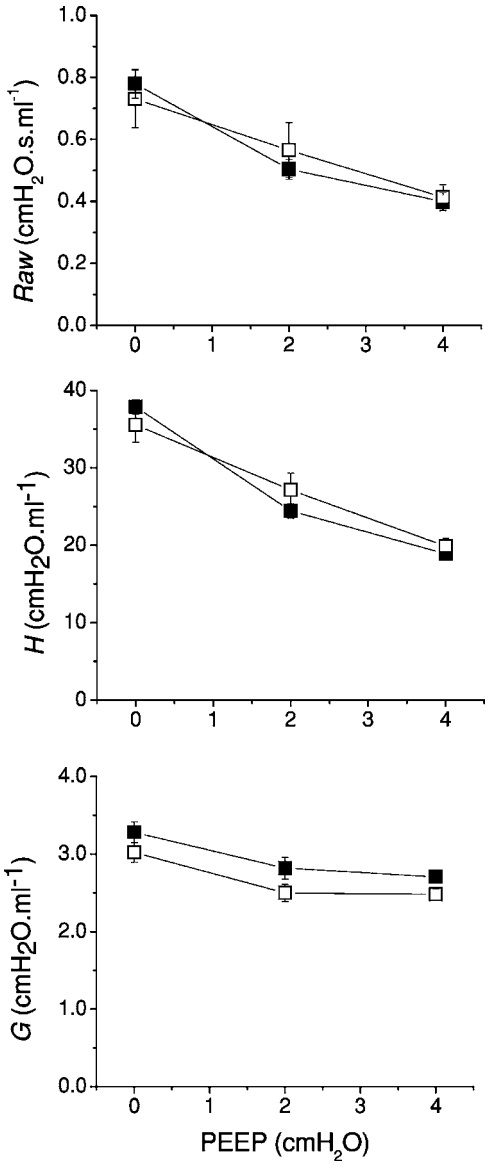

FIGURE 5. Parameters of the constant phase model of respiratory impedance (Eq. 8) in mice ($n = 8$) before (solid symbols) and after (open symbols) paralysis. *Raw*, *H*, and *G* reflect airway resistance, tissue stiffness, and tissue damping, respectively. Error bars indicate SEM.

to paralyze the animal lest muscular activity disturb the measurements.[1] Consequently, for the present study we chose to replace the conventional role of the respiratory muscles with an external mechanism for achieving gas compression, while avoiding any effect on chamber pressure from the compressive mechanism itself. This precluded the use of a compression system based on the linear reciprocating motion of any kind of plunger operated from outside the chamber, because changes in the length of the plunger inside the chamber would have caused changes in chamber pressure and so would have obscured those changes due to gas compression that we sought to identify. We thus

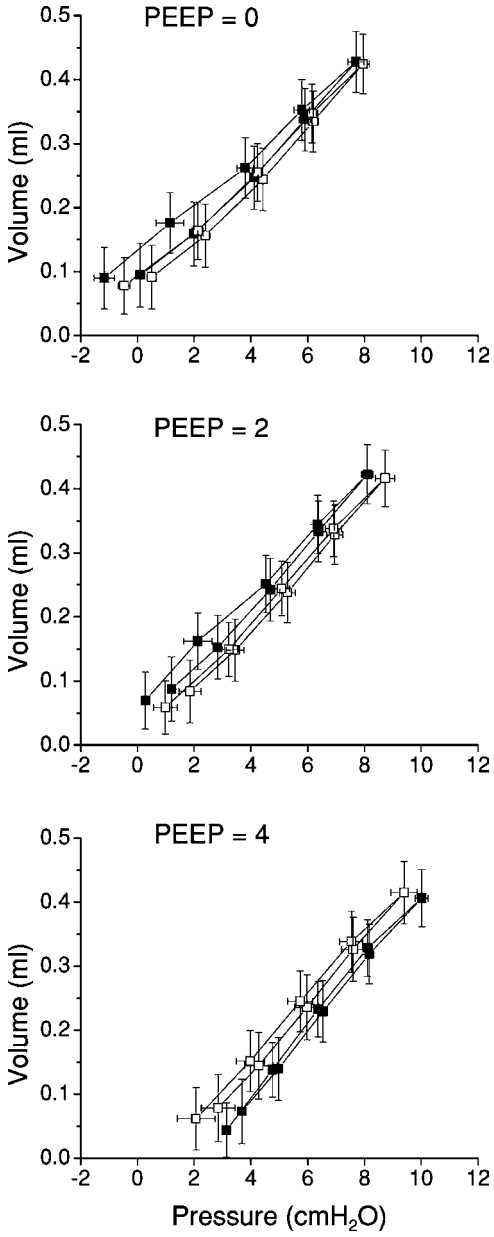

FIGURE 6. Quasi-static *PV* curves from paralyzed and nonparalyzed mice ($n = 8$) at three different PEEP levels. Loops proceed in a counterclockwise direction beginning with inspiration. Error bars indicate SEM.

TABLE 1. Average *E* calculated from *PV* curves in Fig. 6.

PEEP	Nonparalyzed *E* (mean ± SEM)	Paralyzed *E* (mean ± SEM)
0	25.5 ± 0.43	23.0 ± 0.57[a]
2	21.2 ± 0.62	20.6 ± 0.77[a]
4	18.2 ± 0.50	19.6 ± 1.04[a]

Note. $p > 0.05$, one-way ANOVA.
[a]Not statistically different from nonparalyzed.

utilized a rotating shaft because it could be operated without changing the amount of shaft material inside the chamber.

We first validated our mouse plethysmograph by measuring V_{TG} in the conventional way using spontaneous breathing efforts and comparing them to an independent assessment of V_{TG} based on Archimedes' principle. These measurements agreed closely (Fig. 4). Furthermore, the V_{TG} measurements increased with PEEP in almost exactly the same way as lung volume increased with pressure during the quasi-static *PV* measurements (Fig. 6). To confirm this observation we fitted a straight line to the mean V_{TG} measurements in Fig. 4 obtained at PEEP = 2 and 4 cmH_2O and divided the result into the difference in PEEP to get an estimate of E. The values obtained were 18.9 and 17.9 $cmH_2O.ml^{-1}$ in paralyzed and nonparalyzed mice respectively, which closely matches the E calculated from the *PV* curves (Table 1). This supports the validity of our V_{TG} measurements made during spontaneous breathing, and so support their use as a basis for comparison against the measurements made by chest compression.

V_{TG} has been measured in mice by other workers. For example, V_{TG} measured by inflating degassed lungs has been reported to be 0.27 and 0.37 ml in A/J and C3H/HeJ mice, respectively[15] and a CT scanning technique gave values ranging from 0.35 to 0.4 ml in A/J mice and 0.25 to 0.3 ml in C3H/HeJ mice12. Also, our measurements of V_{TG} in spontaneously breathing BALB/c mice ($V_{TG} = 0.22 \pm 0.01$ ml, PEEP = 0) are slightly less than those reported for C57BL/6 mice ($V_{TG} = 0.25 \pm 0.01$ ml) by Tankersley *et al.*[15] who inflated degassed lungs with air until they reached functional residual capacity. Thus, while our measurements of V_{TG} are similar to those described by others, there is some variation with measurement technique and strain of mouse.

The chest compression technique produced sufficient compression of thoracic gas that we were able to clearly identify the relationship between P_{ao} and P_b (Fig. 2). However, we found that the mean slope of this relationship gave values for V_{TG} that were 1.4 to 2.3 times higher than those obtained in spontaneously breathing mice. Furthermore, a plot of P_{ao} versus P_b revealed a substantial degree of looping that was not present in the spontaneous breathing data (Fig. 2). We initially considered that this might be due to compression of bowel gas that somehow did not occur when the animals breathed spontaneously. However, we found the same looping when we compressed an animal with an open abdomen, or even with an open chest, and injection of air into the colon did not affect our measurements (results not shown). We then wondered if the looping between P_{ao} and P_b could be due to inhomogeneous compression of the lungs leading to regional variations in alveolar pressure. We have previously measured V_{TG} in mice following saline lung lavage and observed substantial

looping between P_{ao} and P_b during spontaneous breathing efforts,[10] which we attributed to heterogeneous mechanical behavior throughout the lung resulting from the induced injury. However, in the present study there was no change in H or any other parameters of lung mechanics when the animals were paralyzed (Fig. 5). Furthermore, regional heterogeneities of lung function would be expected to lead to more pronounced looping both at lower PEEP levels and when the frequency of compression was increased, neither of which we observed. We therefore conclude that mechanical heterogeneities of ventilation were not responsible for the looping.

The slow recovery of P_b at the expiratory end of the loop during chest compression (Fig. 2) was not mirrored by a corresponding change in P_{ao}, indicating that the phenomenon responsible for the looping was taking place in the plethysmograph chamber rather than in the lungs themselves. We also observed that we could reduce the looping if we introduced a heat-sink in the form of copper wool wrapped around the mouse (data not shown). We believe that the force applied with the paddle released the heated air trapped in the fur, such an effect would be absent in spontaneously breathing animals and would explain why no looping was observed during inspiratory efforts. By removing the air trapped in the fur of the animals, when soaked in water, we substantially reduced the looping of the P_{ao} and P_b plot (Fig. 3). Consequently, we finally settled on the notion that thermal effects in the chamber were responsible for the looping. This presented the possibility of correcting the looping by accounting for the dynamics of the thermal effects and removing them from P_b. We therefore developed a model of heat transfer from the animal to the walls of the chamber that accounted for the transient changes in P_b we observed each time the paddle was lifted from the animal's chest (Eq. 7).

Using Eq. 7 to correct for the thermal artifacts at PEEPs of 2 and 4 cmH_2O resulted in values for V_{TG} that closely matched those obtained during spontaneous breathing (Figs. 2 and 4). At a PEEP of 0 cmH_2O, however, V_{TG} obtained by chest compression was still higher than that obtained from spontaneous breathing efforts (Fig. 4). We do not know how to explain this, but speculate that the partial closure of lung units likely to occur at such a low PEEP might have affected transmission of pressure from the thoracic gas to its measurement point at the airway opening. In any case, measuring V_{TG} with our chest compression technique in paralyzed mice appears to be valid at PEEP levels of 2 cmH_2O and higher.

In summary, we have developed a plethysmographic method for measuring V_{TG} in mice by externally compressing the chest and correcting for thermal artifacts due to release of heat from the animal into the plethysmograph chamber. The values of V_{TG} obtained agree closely with those obtained using the conventional approach of having the animal breathe spontaneously against a closed airway at PEEP levels of 2 cmH_2O and greater. Our method will allow V_{TG} to be measured in mice in those situations where paralysis is required for the precise measurement of lung function or when spontaneous breathing is weak or absent.

ACKNOWLEDGMENTS

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