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The ventilatory depressant actions but not the antinociceptive effects of morphine are blunted in rats receiving intravenous infusion of L-cysteine ethyl ester

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Abstract

This study demonstrates that intravenous infusion of the cell-penetrant thiol ester, L-cysteine ethyl ester (L-CYSee), to adult male Sprague-Dawley rats elicited (a) minor alterations in frequency of breathing, expiratory time, tidal volume, minute ventilation, or expiratory drive but

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Conflict of Interest Statement

The authors declare that they have no competing financial interests or personal relationships that would have influenced the studies that are described in this manuscript.

pronounced changes in inspiratory time, end-inspiratory and expiratory pauses, peak inspiratory and expiratory flows, EF_{50} , relaxation time, apneic pause, inspiratory drive and non-eupneic breathing index, (b) minimal changes in arterial blood-gas (ABG) chemistry (pH, pCO_2 , pO_2 , SO_2) and Alveolar-arterial (A-a) gradient (index of alveolar gas exchange), and (c) minimal changes in antinociception (tail-flick latency). Subsequent injection of morphine (10 mg/kg, IV) elicited markedly smaller effects on the above parameters, ABG chemistry, and A-a gradient in rats receiving L-CYSee, whereas morphine antinociception was not impaired. Infusions of L-cysteine or L-serine ethyl ester (oxygen rather than sulfur moiety), did not affect morphine actions on ABG chemistry or A-a gradient. L-CYSee (250 μ mol/kg, IV) injection elicited dramatic changes in ventilatory parameters given 15 min after injection of morphine in rats receiving L-CYSee. Our findings suggest that (a) L-CYSee acts in neurons that drive ventilation, (b) L-CYSee reversal of the adverse actions of morphine on ventilation, ABG chemistry and A-a gradient may be via modulation of intracellular signaling pathways activated by morphine rather than by direct antagonism of opioid receptors since morphine antinociception was not diminished by L-CYSee, and (c) the thiol moiety of L-CYSee is vital to efficacy, (d) intracellular conversion of L-CYSee to an S-nitrosylated form may be part of its mechanism of action.

Keywords

L-thiol ester infusion; Morphine; Ventilatory depression; Arterial blood-gas chemistry; Analgesia; Sprague-Dawley rats

1. Introduction

The clinical effectiveness of opioids as analgesics is often compromised by their ability to induce adverse effects on ventilatory parameters [1–6]. Although the administration of opioid receptor (OR) antagonists can in many cases reverse opioid-induced respiratory depression (OIRD), these antagonists also diminish the analgesic/antinociceptive actions of opioids, which may not be a problem in overdose situations, but which is contraindicated when analgesia is required during and after injuries and surgical procedures [2–4]. Over recent years, numerous classes of drugs that do not directly block ORs have been examined for their potential to overcome OIRD while preserving analgesia [2–8]. Most of these drugs did not reach the level of consideration for clinical trials in humans or did not meet expected therapeutic outcomes in such trials because of a lack of efficacy and/or unacceptable levels of toxicity or side effects [4,5,7,8]. The current opioid crisis has reinforced the urgent necessity to develop drugs that can effectively reverse OIRD by mechanisms independent of OR blockade for numerous reasons including that OR antagonists can elicit often life-threatening withdrawal symptoms in habitual opioid users [5,6].

Membrane-permeable thioesters such as L-cysteine ethyl ester (L-CYSee), D-cysteine ethyl ester (D-CYSee), L-cysteine methyl ester (L-CYSme), L-glutathione ethyl ester (L-GSHee), D-cystine dimethyl ester (D-CYSdime) and D-cystine diethyl ester (D-CYSdiee) readily gain access to central and peripheral tissues and have diverse pharmacological actions [9–14]. We have reported that these L-, D-thioesters prevent and/or reverse the adverse effects of opioids such as morphine and fentanyl on ventilation, arterial blood-gas (ABG)

chemistry, and Alveolar-arterial (A-a) gradient (index of alveolar gas exchange in the lungs) in unanesthetized freely-moving rats whereas L- and D-thioesters minimally impact the sedative or analgesic effects of the opioids [10–14]. In contrast, the parent thiols, L- or D-cysteine, L-glutathione, and D-cystine, only minimally altered the effects of the opioids [9–14]. In initial studies that were designed to better understand structure-activity relationships of L- and D-thioesters, we demonstrated that N-acetyl-L-cysteine methyl ester [14] and L-methionine ethyl ester (unpublished observations) could not reverse any of the deleterious effects of morphine on breathing in unanesthetized rats. These findings suggest that modifications to the L-,D-cysteine backbone or sulfur moiety prevent L-,D-thioester efficacy. The mechanisms of action of these L-, D-thioesters could involve (i) direct interactions with yet to be determined functional proteins, (ii) binding to the putative L-,D-cysteine binding protein, myristoylated alanine-rich C-kinase substrate [15], (iii) interruption of OR- β -arrestin-coupled cell signaling processes to spare the analgesic G-protein-dependent actions of morphine [16,17], and/or (iv) conversion of reduced L-,D-thioesters to S-nitrosothiols via S-nitrosylation of the sulfur atom by nitric oxide synthase-dependent processes [15–21], which may act similarly S-nitroso-L-CYSee [22].

Our studies to date in freely-moving adult rats have only examined the effects of bolus intravenous injections of L-,D-thioesters against the ventilatory depressant effects of opioids [9–14]. We subsequently wanted to extend our knowledge about the pharmacological profile and efficacy of these L-,D-thioesters by determining whether continuous intravenous infusion of these agents would be efficacious against OIRD while sparing opioid analgesia. We began these studies by determining whether the effects of a bolus injection of morphine on ventilatory parameters, ABG chemistry, A-a gradient, and antinociception were altered in freely-moving adult male Sprague-Dawley rats receiving continuous intravenous infusion of L-CYSee. To extend our structure-activity knowledge about L-,D-thioesters, we also determined whether similar infusions of the cell penetrant L-thioester, L-serine ethyl ester (L-SERee) [9], which has an oxygen instead of a sulfur atom, could be as equally effective as L-CYSee in reversing morphine OIRD.

2. Methods

2.1. Permissions, rats, and surgical procedures

All studies were done in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH Publication No. 80–23) revised in 1996 and in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (<http://www.nc3rs.org.uk/page.asp?id=1357>). All protocols were approved by the Animal Care and Use Committees of Galleon Pharmaceuticals, the University of Virginia Case, and Case Western Reserve University. Adult male Sprague Dawley rats were purchased from *Harlan Industries* (Madison, WI, USA). After five days of recovery from transportation, the rats received femoral artery catheters and/or two jugular vein catheters under 2–3% isoflurane anesthesia [10–14, 23]. One of the jugular catheters was to allow for continuous infusion of L-CYSee or L-SERee and the other was to allow bolus injections of morphine (10 mg/kg, IV). The rats were given 4 days to recover from surgery. All femoral arterial catheters were flushed daily with a heparin solution (50 units in 0.1 M, pH 7.4 phosphate-buffered saline). On

the day of the study, the arterial catheters were flushed with 0.3 ml of phosphate-buffered saline (0.1 M, pH 7.4) 2–3 h before the start of the protocols. The pH of all stock solutions of vehicle, L-CYSee, and L-SERee was adjusted to a pH of 7.3 with 0.125 M NaOH. All studies were done in a quiet laboratory room with a relative humidity of $49 \pm 3\%$ and room temperature of 21.3 ± 0.2 °C. The ventilatory studies, ABG chemistry studies, and antinociception studies were performed in separate groups of rats to not compromise each set of recordings. The plethysmography and antinociception recording sessions and arterial blood sampling studies (ABG assays) were done by one investigator whereas another filled the syringes with test agents, such that the investigator performing the experiment was blind to the protocol. In addition, the data files resulting from each experiment were collated and analyzed by yet another investigator in the group.

2.2. Protocols for whole-body plethysmography measurement of ventilatory parameters

Ventilatory parameters were recorded continuously in unrestrained freely-moving rats by a whole-body plethysmography system (PLY3223; Data Sciences International, St. Paul, MN) as described previously [9–14]. Directly recorded and derived parameters (see 53–56) are defined in Table 1. These parameters and their abbreviations are: frequency of breathing (Freq), tidal volume (TV), minute ventilation (MV), inspiratory time (Ti), expiratory time (Te), Ti/Te, end-inspiratory pause (EIP), end-expiratory pause (EEP), relaxation time (RT), peak inspiratory flow (PIF), peak expiratory flow (PEF), expiratory flow at 50% expired TV (EF₅₀), point in expiration when PEF occurs expressed as a fraction of Te (R_pef), inspiratory drive (TV/Ti), expiratory drive (TV/Te), apneic pause [(Te/RT)–1], non-eupneic breathing index (NEBI) and NEBI corrected for Freq (NEBI/Freq). On the day of the study, each rat was placed in an individual plethysmography chamber and given 60–75 min to acclimatize so that baseline (pre) ventilatory parameter values could be defined. One group of rats (n = 5) received a continuous infusion of vehicle (20 µL/min, IV) and another received a continuous infusion of L-CYSee (14.3 µmol/kg/min, IV). Both infusions were maintained throughout the study. After 35 min of infusion, all rats received a bolus injection of morphine (10 mg/kg, IV). After, a further 15 min, the vehicle-infused rats received a bolus injection of vehicle whereas the L-CYSee-infused rats received a bolus injection of L-CYSee (250 µmol/kg, IV) and ventilatory parameters were recorded for a further 45 min. The body weights of the two groups were equivalent to one another (see Table 2) and as such, ventilatory parameters related to volumes such as TV, PIF, PEF, and EF₅₀ are presented without correcting for body weight. FinePointe (DSI) software constantly corrected digitized ventilatory values originating from the respiratory waveforms for alterations in chamber humidity and temperature. Pressure changes associated with the respiratory waveforms were converted to volumes (e.g., TV, PIF, PEF, EF₅₀) using the algorithms of Epstein and colleagues [24,25]. Factoring in chamber humidity and temperature, cycle analyzers filtered the acquired signals, and FinePointe algorithms generated an array of box flow data that identified waveform segments as acceptable breaths, and minimum and maximum values were determined. Flows at this point were box-flow signals and from this array, minimum and maximum box flow values were then determined and multiplied by a compensation factor from the algorithms [24,25] thus producing TV, PIF, PEF, and EF₅₀ values used to determine non-eupneic breathing events expressed as

NEBI, reported as the percentage of non-eupneic breathing events per each individual epoch [10–14, 23, 26]. Apneic pause was determined by the formula, $(Te/RT) - 1$ [10–14, 23].

2.3. Protocols for blood gas measurements and determination of Arterial-alveolar gradient

ABG chemistry parameters (pH, pCO₂, pO₂, sO₂) and A-a gradients were recorded before (Pre) and 30 min after commencing continuous infusions of vehicle (20 µL/kg/min, IV; n = 9 rats, 84.4 ± 0.4 days of age; 341 ± 3 g), L-CYSee (7.15 µmol/kg, IV; 84.7 ± 0.3 days; 346 ± 3 g) or L-SERee (7.15 µmol/kg, IV; 84.3 ± 0.4 days; 341 ± 3 g) as described previously [9–14, 23]. All rats received a bolus injection of morphine (10 mg/kg, IV) and arterial blood samples (100 µL) were taken after 5, 15, 30, 45, and 60 min. The above samples of arterial blood were injected into a Radiometer blood-gas analyzer (ABL800 FLEX) to obtain ABG values. The A-a gradient defines the differences between alveolar O₂ and arterial blood O₂ concentrations (see 10–14, 23). A fall in PaO₂, without change in A-a gradient, is due to hypoventilation, whereas a decrease in PaO₂ with a concomitant elevation in A-a gradient indicates an ongoing mismatch in ventilation-perfusion in alveoli [10–14, 23]. A-a gradient = PAO₂ – PaO₂, where PAO₂ is the partial pressure (p) of alveolar O₂ and PaO₂ is pO₂ in the sampled arterial blood. $PAO_2 = [(FiO_2 \times (P_{atm} - P_{H_2O})) - (PaCO_2 / \text{respiratory quotient})]$, where FiO₂ is the fraction of O₂ in inspired air; P_{atm} is atmospheric pressure; P_{H₂O} is the partial pressure of H₂O in inspired air; PaCO₂ is pCO₂ in arterial blood; and respiratory quotient (RQ) is the ratio of CO₂ eliminated/O₂ consumed. We took FiO₂ of room-air to be 21% = 0.21, P_{atm} to be 760 mmHg, and P_{H₂O} to be 47 mmHg. We took the RQ value of our adult male rats to be 0.9 [10–14, 23, 27, 28].

2.4. Antinociception assessment by tail-flick latency assay

We recorded TFL values before (–15 and 0 min, Pre values), and at 15 and 35 min during the continuous infusion of vehicle (20 µL/kg/min, IV; 81.9 ± 0.3 days; 325 ± 3 g), L-CYSee (14.3 µL/kg/min, IV; 82.2 ± 0.4 days; 327 ± 3 g) or L-SERee (14.3 µmol/kg/min, IV; 81.8 ± 0.2 days; 323 ± 3 g). Both groups of rats received an injection of morphine (10 mg/kg, IV) after the TFL recording at 35 min of infusion, and TFL values were recorded again after 15, 30, and 60 min. TFL values were determined using a Tail-Flick Analgesia Meter (IITC Life Science Inc., USA) as detailed previously [10–14, 23]. The procedure involved a minor degree of manual restraint while positioning the tail to apply a thermal beam sufficient to induce a latency of tail withdrawal of approximately 2.5 s. TFL data are shown as actual TFL (sec) and as maximum possible effect (%MPE) determined by the formula, $\%MPE = [(post\text{-}injection\ TFL - baseline\ TFL) / (12 - baseline\ TFL)] \times 100$ [10–14, 23].

2.5. Data analyses

Directly recorded and arithmetically-derived ventilatory parameters (1 min bins) were taken for statistical analyses. Pre-drug 1 min bins excluded occasional marked deviations from resting values due to abrupt rat movements such as scratching. All ventilatory, ABG chemistry, A-a gradient, and TFL are presented as mean ± SEM and were evaluated using one-way and two-way ANOVA followed by Bonferroni corrections for multiple comparisons between means using the error mean square terms from each ANOVA analysis [29–32] as detailed previously [9–14]. A $P < 0.05$ value denoted an initial level of statistical

significance that was modified according to the number of comparisons between means as described by Wallenstein et al. [31]. The modified t -statistic is $t = (\text{mean group 1} - \text{mean group 2})/[s \times (1/n_1 + 1/n_2)^{1/2}]$ where s^2 = the mean square within groups term from the ANOVA (the square root of this value is used in the modified t -statistic formula) and n_1 and n_2 are the number of rats per group being compared. Based on elementary inequality called Bonferroni's inequality, a conservative critical value for modified t -statistics from tables of t -distribution using a significance level of P/m , where m is the number of comparisons between groups to be performed [32]. The degrees of freedom are those of the mean square for within-group variation from ANOVA tables. The critical Bonferroni value cannot be found in conventional tables of the t -distribution but can be approximated from tables of the normal curve by $t^* = z + (z + z^3)/4n$, with n being the degrees of freedom and z being the critical normal curve value for P/m [29–32]. Wallenstein et al. [31] first demonstrated that the Bonferroni procedure is preferable for general use since it has the widest range of applications and because it provides critical values that are lower than those of other procedures when the investigator can limit the number of comparisons (and will be slightly larger than other procedures if many comparisons are made. As mentioned, a value of $P < 0.05$ was taken as the initial level of statistical significance [29–31] and the statistical analyses were performed with the aid of GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Ventilatory responses elicited by morphine in rats receiving infusion of L-CYSee

Baseline parameters in the two groups of rats prior to them receiving infusions of vehicle or L-CYSee are summarized in Table 1. As can be seen, there were no between-group differences for any parameter ($P > 0.05$, for all comparisons). Freq, TV, and MV values during the various stages of the experiment are summarized in the left-hand panels of Fig. 1. The infusion of L-CYSee (14.3 $\mu\text{mol/kg/min}$, IV) elicited relatively minor increases in Freq (Panel A) and minor decreases in TV (Panel C) such that there were only trivial changes in MV (Panel E). A bolus injection of morphine (10 mg/kg, IV) into rats receiving vehicle infusion elicited a decrease in Freq and more sustained decreases in TV and MV. In rats receiving an infusion of L-CYSee, the injection of morphine elicited a pronounced increase in Freq, a smaller but distinct reduction in TV than in vehicle-infused rats such that morphine elicited trivial decreases in MV. The injection of L-CYSee (250 $\mu\text{mol/kg}$, IV) given 15 min after the injection of morphine elicited an increase in Freq and TV resulting in a sustained increase in MV. The right-hand panels summarize the sum of the responses during each phase of the protocol. Infusion (columns designated as INF) of L-CYSee elicited an increase in Freq (Panel B), a decrease in TV (Panel D) and no change in MV (Panel F). The total decreases in Freq elicited by morphine in vehicle-infused rats (first 15 min, columns designated as MOR) were converted to pronounced increases in the L-CYSee-infused rats. The total decreases in TV elicited by morphine were much smaller in the L-CYSee-infused rats and as such the total changes in MV were not significant. The injection of L-CYSee (250 $\mu\text{mol/kg}$, IV) maintained the increase in Freq, restored TV to baseline values resulting in a pronounced total increase in MV.

Ti, Te, and Ti/Te (respiratory quotient) values during the various stages of the experiment are summarized in the left-hand panels of Fig. 2. The infusion of L-CYSee elicited a substantial decrease in Ti (Panel A) and minor changes in Te (Panel C) resulting in a decrease in Ti/Te (Panel E). Injection of morphine into vehicle-infused rats elicited a sustained increase in Ti, a transient increase followed by a sustained increase in Te, and a sustained increase in Ti/Te. In rats receiving L-CYSee, the injection of morphine elicited an increase in Ti, a transient increase followed by a sustained decrease in Te resulting in a gradual and sustained increase in Ti/Te paralleling that in vehicle-infused rats. Subsequent bolus injection of L-CYSee did not exert substantial changes in these parameters. With respect to the total changes in each parameter, the infusion (columns INF) of L-CYSee elicited a decrease in Ti (Panel B), no change in Te (Panel D), and therefore a decrease in Ti/Te (Panel F). Total increases in Ti elicited by morphine in vehicle-infused rats (first 15 min, MOR columns) were converted to increases in L-CYSee-infused rats. Total increases in Te elicited by morphine were converted to decreases in L-CYSee-infused rats and as such, the total changes in Ti/Te were not different from those in vehicle-infused rats. The injection of L-CYSee (250 $\mu\text{mol/kg}$, IV) maintained the changes in Ti, Te and Ti/Te elicited by morphine in L-CYSee-infused rats.

EIP and EEP values are summarized in the left-hand panels of Fig. 3. Infusion of L-CYSee elicited a substantial decrease in EIP (Panel A) but a substantial and sustained increase in EEP (Panel C). Injection of morphine into vehicle-infused rats elicited a sustained increase in EIP and a transient increase in EEP. In rats receiving L-CYSee, morphine elicited an increase in EIP that was similar to vehicle-infused rats. Morphine also elicited pronounced and sustained decrease in EEP. The subsequent bolus injection of L-CYSee elicited minor changes in these parameters. With respect to the total changes in EIP and EEP, the infusion (columns INF) of L-CYSee elicited a decrease in EIP (Panel B) but a large increase in EEP (Panel D). The total increases in EIP elicited by morphine in vehicle-infused rats (first 15 min, MOR columns) were absent in L-CYSee-infused rats. The total increases in EEP elicited by morphine were not greatly affected by L-CYSee-infusion. The injection of L-CYSee (250 $\mu\text{mol/kg}$, IV) did not further influence EIP or EEP.

PIF, PEF, and Rpef values are summarized in the left-hand panels of Fig. 4. The infusion of L-CYSee elicited substantial increases in PIF (Panel A) and PEF (Panel C) and substantial and sustained decreases in Rpef (Panel E). Injection of morphine into vehicle-infused rats caused sustained decreases in PIF, a transient decrease in PEF, and restored Rpef values to pre-morphine values. In L-CYSee-infused rats, the injection of morphine elicited a pronounced decrease in PIF and PEF to pre-morphine levels whereas it caused a return of Rpef to pre-morphine values. The subsequent injection of L-CYSee elicited relatively minor but sustained increases in PIF, PEF, and Rpef. With respect to the total changes in each parameter, the infusion of L-CYSee (columns INF) elicited pronounced increases in PIF (Panel B) and PEF (Panel D) and a decrease in Rpef (Panel F). The total increases in PIF and PEF elicited by morphine in vehicle-infused rats (first 15 min, MOR columns) were converted to increases in PIF and PEF in L-CYSee-infused rats. The total increases in Te elicited by morphine were similar in the two groups. The injection of L-CYSee maintained the effects of L-CYSee infusion on PIF and PEF and augmented the effects of the infusion on Rpef.

EF₅₀, Relaxation Time, and Apneic Pause values are summarized in the left-hand panels of Fig. 5. The infusion of L-CYSee elicited a substantial increase in EF₅₀ (Panel A), a substantial decrease in Relaxation Time (Panel C), and a substantial increase in Apneic Pause (Panel E). The injection of morphine into vehicle-infused rats elicited a transient decrease in EF₅₀, a sustained decrease in Relaxation Time, and transient increases in Apneic Pause. In L-CYSee-infused rats, the injection of morphine elicited a pronounced decrease in EF₅₀ to values still above pre-morphine levels whereas it had minimal initial effects on Relaxation Time. The injection of morphine elicited a pronounced and sustained decrease in Apneic Pause values back to pre-L-CYSee-infusion values. Subsequent bolus injection of L-CYSee elicited a minor but sustained increase in EF₅₀, and minor changes in Relaxation Time and Apneic Pause. Regarding the total changes in each parameter, the infusion of L-CYSee (columns INF) elicited pronounced increases in PIF (Panel B), decreases in Relaxation Time (Panel D), and increases in Apneic Pause (Panel F). The total increases in EF₅₀ and decreases in Relaxation Time elicited by L-CYSee infusion were sustained following the injections of morphine and L-CYSee. The total increase in Apneic Pause elicited by the infusion of L-CYSee was diminished by the injection of morphine and absent upon bolus injection of L-CYSee.

Inspiratory Drive (TV/Ti) and Expiratory Drive (TV/Te) values are summarized in the left-hand panels of Fig. 6. L-CYSee infusion elicited substantial increases in Inspiratory Drive (Panel A) but minor changes in Expiratory Drive (Panel C). The injection of morphine into vehicle-infused rats caused sustained decreases in Inspiratory Drive and transient decreases in Expiratory Drive. In L-CYSee-infused rats, morphine elicited a rapid decrease in Inspiratory Drive toward pre-morphine levels whereas it caused an initial decrease followed by a sustained increase in Expiratory Drive. Subsequent injection of L-CYSee elicited a relatively minor but sustained increase in Inspiratory Drive and robust and sustained increases in Expiratory Drive. With respect to the total changes in each parameter, the of L-CYSee infusion (columns INF) elicited pronounced increases in Inspiratory Drive (Panel B) but minimal changes in Expiratory Drive (Panel D). The total increases in Inspiratory and Expiratory Drive elicited by morphine in vehicle-infused rats (first 15 min, MOR columns) were absent in L-CYSee-infused rats. The injection of L-CYSee maintained the effects of L-CYSee infusion on Inspiratory Drive but elicited a sustained increase in Expiratory Drive.

NEBI and NEBI/Freq values during the various stages of the experiment are summarized in the left-hand panels of Fig. 7. The infusion of L-CYSee elicited substantial decreases in NEBI (Panel A) and NEBI/Freq (Panel C). The injection of morphine into vehicle-infused rats elicited a rapid and pronounced increase in NEBI and NEBI/Freq that rapidly decreased toward zero levels. In L-CYSee-infused rats, the injection of morphine did not affect the markedly diminished NEBI and NEBI/Freq values. The subsequent injection of L-CYSee elicited minimal responses in either group. Regarding the total changes in NEBI and NEBI/Freq, the infusion of L-CYSee (columns INF) elicited pronounced falls in NEBI (Panel B) and NEBI/Freq (Panel D) that were minimally affected by the subsequent bolus injections of morphine or L-CYSee.

ABG chemistry parameters (pH, pCO₂, pO₂, and sO₂) during the experiments with L-CYSee and L-SERee are summarized in Fig. 8. The infusion of L-CYSee (7.15 μmol/kg, IV)

or L-SERee (7.15 $\mu\text{mol/kg}$, IV) did not (as recorded after 30 min of infusion) affect pH (Panel A), pCO_2 (Panel B), pO_2 (Panel C) and sO_2 (Panel D). The injection of morphine (10 mg/kg, IV) elicited large and sustained reductions in pH, pO_2 , and sO_2 that were accompanied by equally pronounced increases in pCO_2 in vehicle-infused rats. The ABG chemistry values following injection of morphine in rats receiving L-SERee were very similar to those in vehicle-infused rats. In contrast, the changes in pH, pCO_2 , pO_2 , and sO_2 were dramatically diminished in rats receiving the infusion of L-CYSee. A-a gradient values during the L-CYSee and L-SERee studies are summarized in Fig. 9. The infusion of L-CYSee (7.15 $\mu\text{mol/kg}$, IV) or L-SERee (7.15 $\mu\text{mol/kg}$, IV) did not (as recorded after 30 min of infusion) affect A-a gradient. The injection of morphine (10 mg/kg, IV) elicited large and sustained increases in A-a gradient that were of similar magnitude in vehicle-infused and L-SER-infused rats. In contrast, the changes in A-a gradient were markedly reduced in rats receiving L-CYSee infusion.

TFL values during studies with L-CYSee and L-SERee are summarized in Fig. 10. The injection of morphine (10 mg/kg, IV) elicited sustained increases in TFL that were of similar magnitude in rats receiving infusions of vehicle, L-SERee (14.3 $\mu\text{mol/kg/min}$, IV), and L-CYSee (14.3 $\mu\text{mol/kg/min}$, IV). An important note is that the injection of morphine (10 mg/kg, IV) to vehicle-infused rats caused immediate sedation with the rats most often lying still on their sides or belly. It took about 65–75 min for the rats to regain their feet and move around the chamber. The sedative effects of morphine were similar in rats receiving an infusion of L-CYSee (14.3 $\mu\text{mol/kg/min}$, IV) and the bolus injection of L-CYSee (250 $\mu\text{mol/kg}$, IV).

4. Discussion

This study demonstrates that the deleterious effects of morphine on ventilatory parameters, ABG chemistry, and A-a gradient (alveolar gas exchange) were markedly diminished in rats receiving a continuous intravenous infusion of the highly membrane-permeable, L-thioester, L-CYSee [9, 33–47]. With respect to the plethysmography studies, the infusion rate of L-CYSee (14.3 $\mu\text{mol/kg/min}$, IV) was chosen based on preliminary studies, which determined that it would elicit minimal effects on Freq, TV, and therefore MV. More specifically, this infusion rate was chosen to establish whether the L-thioester could interfere with the ventilatory depressant effects of morphine by processes that are independent of simple stimulation of breathing. Indeed, the present study confirmed that infusion of L-CYSee at 14.3 $\mu\text{mol/kg/min}$ elicited relatively minor increases in Freq, TV, and MV and that the bolus injection of morphine elicited substantially smaller reductions in these parameters in these rats. As such, on face value, it would appear that L-CYSee does interfere with the processes by which morphine decreases Freq and TV and therefore MV.

The finding that L-CYSee infusion did not interfere with the antinociceptive or sedative effects of morphine argues that L-CYSee does not directly block ORs and that the ready access of L-CYSee to peripheral and central sites [36], especially those controlling Freq and TV, modulates the signaling processes that mediate the effects of morphine on breathing. However, the relatively minor effects of L-CYSee infusion on Freq, TV, and MV (the most widely studied of all ventilatory parameters) contrasted with the substantial effects of

L-CYSee infusion on other, less well-reported parameters. Specifically, L-CYSee infusion elicited a fall in T_i without having a major effect on T_e demonstrating that the L-thioester selectively modulates (presumably) central pathways controlling inspiration [48–50]. The observation that L-CYSee elicited robust increases in Inspiratory Drive but no change in Expiratory Drive reinforces the concept that the L-CYSee selectively affects processes controlling inspiratory timing. The ability of L-CYSee to differentially affect ventilatory timing is also evident in that it decreased EIP (reduced the interval between the end of inspiration and the start of expiration) whereas it increased EEP (increased the interval between the end of expiration and the start of inspiration). Moreover, whereas L-CYSee increased Apneic Pause this was due primarily to a pronounced decrease in decreased Relaxation Time rather than T_e . In addition, L-CYSee elicited remarkable increases in PIF, PEF, and EF_{50} , while as would be expected a robust decrease in R_{pef} , clearly suggesting that L-CYSee enhances both inspiratory and expiratory ventilatory mechanics. Whether these effects involve enhancement of neurotransmitter release at neuromuscular junctions and/or enhanced responsiveness of skeletal muscle in the diaphragm and internal and external intercostal muscles remain to be determined.

A remarkable finding was that the L-CYSee infusion elicited a marked drop in ventilatory instability as measured by pronounced falls in NEBI and NEBI/Freq. The low level of baseline NEBI in these (mostly quietly resting) rats is likely due to the occurrence of apneas and type 1 and 2 sighs [10–12, 26] and the ability of L-CYSee to stabilize breathing may involve effects on brainstem and suprapontine pathways that control the ventilatory network [51–53]. With respect to the effects of morphine on ventilatory parameters other than Freq, TV, or MV, it was apparent that L-CYSee infusion markedly blunted the ability of morphine to decrease T_i whereas L-CYSee enhanced morphine-induced decrease in T_e . Morphine reversed the pronounced increase in EEP observed during L-CYSee infusion whereas the ability of morphine to increase EIP was not affected by L-CYSee. Possibly due to the great increases in baseline PIF, PEF, EF_{50} , and Expiratory Drive values observed during the L-CYSee infusion, morphine did not decrease these parameters below baseline levels as in vehicle-infused rats. Morphine had minor effects on the pronounced decrease in Relaxation Time in the L-CYSee-infused rats and restored the substantial increase in Apneic Pause seen during L-CYSee infusion to pre-infusion levels. L-CYSee prevented the initial increase in NEBI elicited by morphine whereas the subsequent and profound decrease in NEBI elicited by morphine occurred in both vehicle- and L-CYSee-infused rats. Finally, the ability of the bolus injection of L-CYSee in rats receiving the L-CYSee infusion to further enhance Freq, TV, MV, PIF, PEF, EF_{50} , Inspiratory Drive, and Expiratory Drive suggests that the L-thioester has enormous potential in overcoming morphine-induced respiratory depression.

An important set of findings was that the ability of morphine (10 mg/kg, IV) to adversely affect ABG chemistry and A-a gradient was markedly diminished in rats that were receiving a lower infusion concentration of L-CYSee (7.15 $\mu\text{mol/kg/min}$) than used in the plethysmography studies (i.e., 50% of the 14.3 $\mu\text{mol/kg/min}$ infusion concentration used in the plethysmography studies). This again demonstrates the obvious ability of L-CYSee to prevent the adverse effects of morphine on breathing and alveolar gas exchange and raises the possibility that even lower concentrations of L-CYSee may have efficacy. A key finding was that the infusion of L-SERee was without effect and as such, it appears that the sulfur

atom of L-CYSee is essential to its activity. There are numerous explanations for these findings including that (i) L-CYSee can bind to functional proteins important for ventilatory control processes and especially those involved in the ability of morphine to depress breathing (see [54–57]) whereas L-SERee cannot, (ii) the degradation of L-CYSee to L-cysteine which then enters numerous metabolic pathways that L-serine cannot [58–66], and (iii) the formation of S-nitroso-L-CYSee [22] and/or S-nitroso-L-cysteine, an endogenous S-nitrosothiol [67–69] with many substantial roles in intracellular signaling cascades (see [70–72]) including those controlling cardiorespiratory function (see [73–77]) and those involved in attenuating OIRD [78,79]. With respect to potential mechanisms by which the infusion of L-CYSee inhibits the adverse effects of morphine on breathing, we have a tentative proposal that (i) morphine-activated μ -ORs recruit histidine triad nucleotide-binding protein 1 (HINT1) while simultaneously activating the neuronal form of NOS (nNOS). HINT1 recruits nNOS to the μ -OR-HINT1 complex [80,81], (ii) an essential process is that nNOS produces small molecule S-nitrosothiols and S-nitrosylated proteins which protect against/reverse morphine-induced respiratory depression, (iii) morphine-activated μ -ORs may recruit HINT1 and initiate the process by which L-CYSee is nitrosylated to S-nitroso-L-CYSee, (iv) such production of S-nitroso-L-CYSee has numerous effects including promoting formation of a μ -OR-NMDA (N-methyl-D-aspartate) receptor super complex, which is a possible mechanism for potentiation of morphine-induced respiratory depression in the absence of S-nitrosothiols [82,83].

A more simplified pathway is presented in the graphical abstract (note that the alpha subunit of G protein is not displayed for the sake of clarity) for why L-CYSee does not affect morphine antinociception/analgesia (upper panel) whereas it does affect the signaling events driving morphine-induced depression of breathing. The upper panel shows that the signaling pathways responsible for morphine-induced antinociception/analgesia involves the ability of morphine to block pain signaling via inhibiting Ca^{2+} -entry into neurons processing nociceptive signals. This involves morphine binding to and activating the μ -OR (Step 1), the $\beta\gamma$ subunits releasing from the receptor complex and binding to and inhibited Ca^{2+} -channels, thereby blocking signal transduction responsible for pain (Step 2) and then β -arrestin (β -arr) binding to the μ -OR triggering endocytosis, and the μ -OR recycling to the cell surface (Step 3). None of these events are modulated by L-CYSee which freely-enters the cell but which cannot bind with efficacy to any of these signaling proteins. The bottom panel depicts how L-CYSee prevents the signaling events driving morphine depression of breathing. The presence of nitric oxide synthase (NOS) in these cells allows for the conversion of L-CYSee to S-nitroso-L-CYSee (SNO-L-CYSee), which binds to the $\beta\gamma$ subunits of the μ -OR upon morphine binding (Step 1). The entry of Ca^{2+} is an essential component of the signaling processes that drive breathing. The binding of SNO-L-CYSee prevents the $\beta\gamma$ subunits from inhibiting Ca^{2+} entry thereby maintaining normal signal transduction processes and preventing morphine suppression of breathing (Step 2). β -arr still binds the complex and triggers endocytosis, but the presence of SNO-L-CYSee prevents recycling to the membrane, reducing available μ -OR at the cell surface (Step 3).

Finally, although it has been established that L-CYSee readily enters peripheral and central tissues following systemic administration to rats [36] and increases levels of L-cysteine and L-cysteine in peripheral tissues in these animals [33], it remains to be established

how L-CYSee affects brain regional levels of L-cysteine, L-cystine and L-glutathione or the functional status of S-glutathionylated proteins, in naïve or morphine-treated rats. Such information will be vital to understand the biochemical effects of L-CYSee and its potential impact on redox homeostasis.

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Data Availability

The datasets generated by this investigation are available freely when requested by contacting the author for correspondence.

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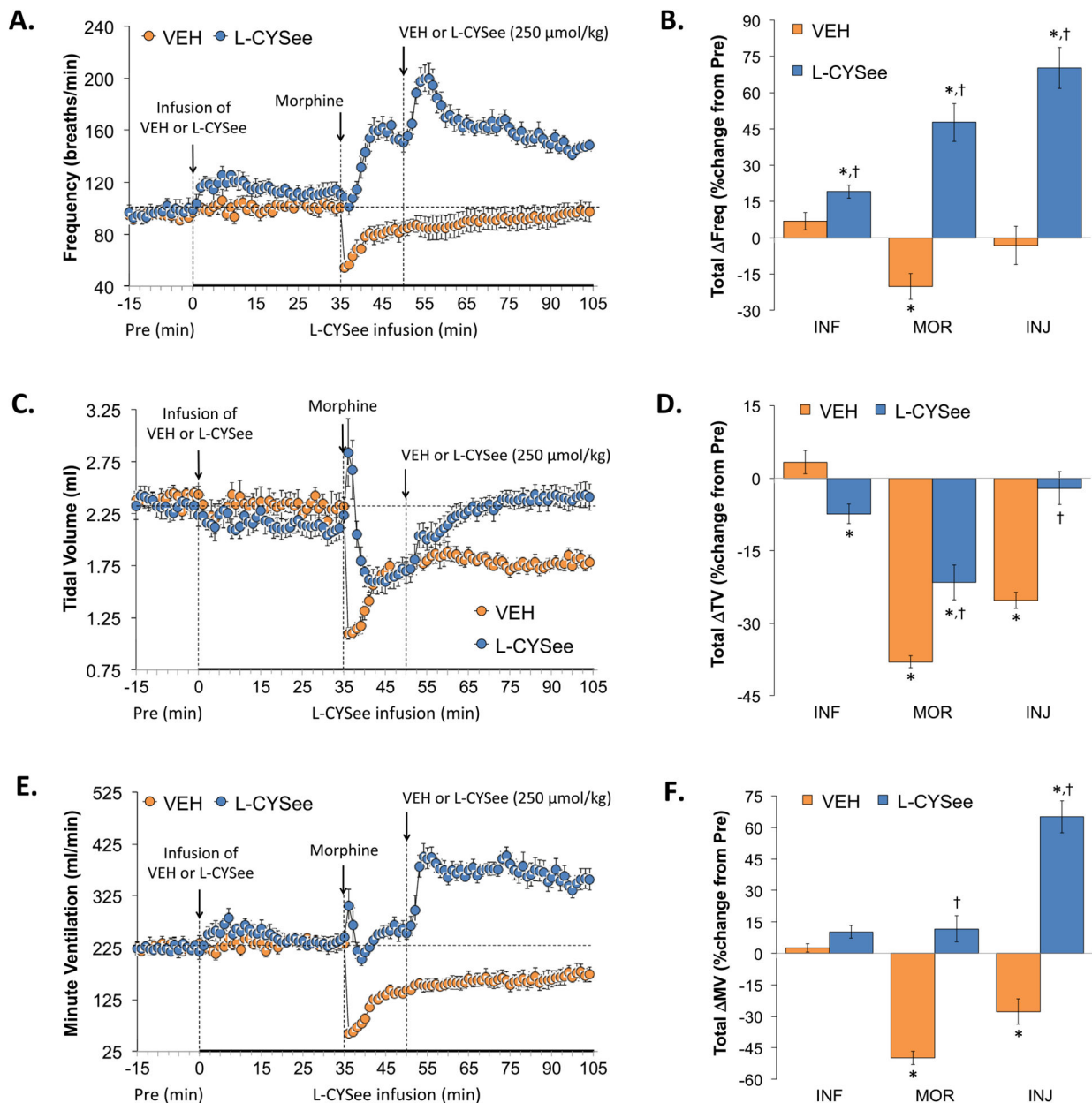
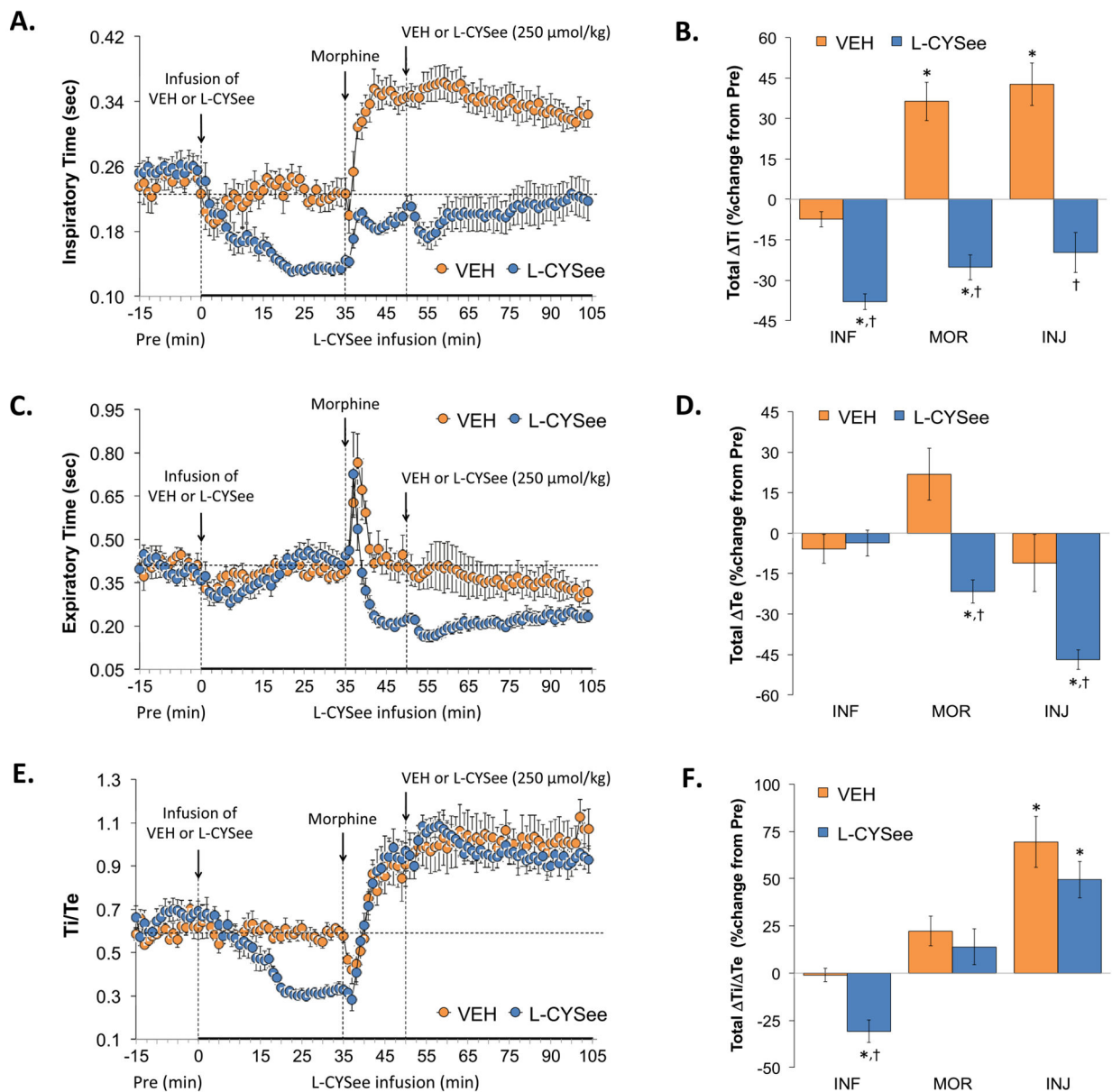


Fig. 1. Changes in frequency of breathing (Freq), tidal volume (TV), and minute ventilation (MV). **Panels A, C, and E:** Actual Freq, TV, and MV values during the various stages of the experiment, respectively. **Panels B, D, and F:** Sum of the Freq, TV, and MV responses recorded during infusion of vehicle (VEH; 20 μ L/min, IV) or L-CYSee (INF; 14.3 μ mol/kg/min, IV) upon injection of morphine (MOR, 10 mg/kg, IV) and following injection of VEH (100 μ L/100 g body weight, IV) or L-CYSee (250 μ mol/kg, IV), respectively. Data are presented as mean \pm SEM. There were 5 rats in the vehicle-infusion group and 6 rats in the L-CYSee-infusion group. * $P < 0.05$, significant change from Pre values. † $P < 0.05$, L-CYSee versus VEH.

**Fig. 2.**

Changes in inspiratory time (Ti), expiratory time (Te), and Ti/Te. **Panels A, C, and E:** Actual Ti, Te, and Ti/Te values during the various stages of the experiment, respectively. **Panels B, D, and F:** Sum of the Ti, Te, and Ti/Te responses recorded during the infusion of vehicle (VEH; 20 μ L/min, IV) or L-CYSee (INF; 14.3 μ mol/kg/min, IV), upon injection of morphine (MOR, 10 mg/kg, IV) and following injection of VEH (100 μ L/100 g body weight, IV) or L-CYSee (250 μ mol/kg, IV), respectively. Data are presented as mean \pm SEM. There were 5 rats in the vehicle-infusion group and 6 rats in the L-CYSee-infusion group. * $P < 0.05$, significant change from Pre values. † $P < 0.05$, L-CYSee versus VEH.

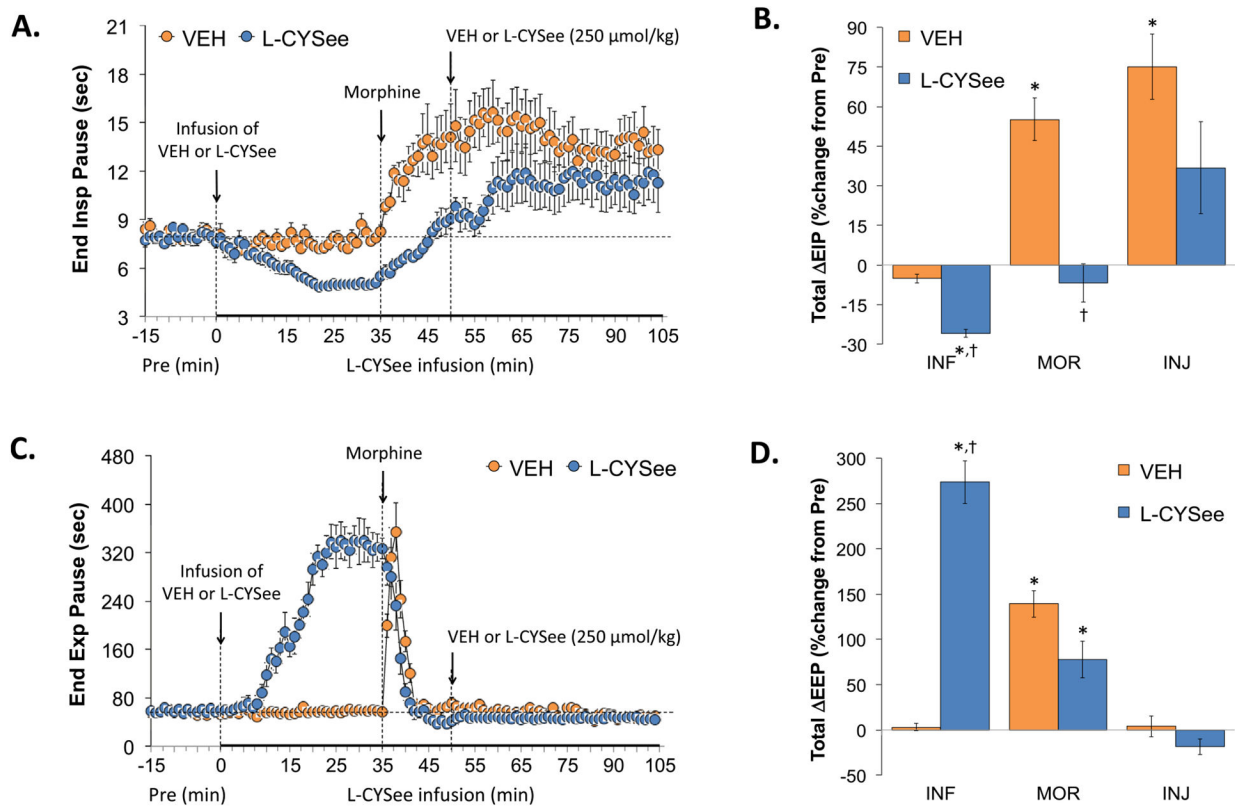


Fig. 3. Changes in end inspiratory pause (EIP) and end expiratory pause (EEP). **Panels A and C:** Actual EIP and EEP values during the various stages of the experiment, respectively. **Panels B and D:** Sum of the EIP and EEP responses recorded during the infusion of vehicle (VEH; 20 μL/min, IV) or L-CYSee (INF; 14.3 μmol/kg/min, IV), upon injection of morphine (MOR, 10 mg/kg, IV) and following injection of VEH (100 μL/100 g body weight, IV) or L-CYSee (250 μmol/kg, IV), respectively. Data are presented as mean ± SEM. There were 5 rats in the vehicle-infusion group and 6 rats in the L-CYSee-infusion group. * $P < 0.05$, significant change from Pre values. † $P < 0.05$, L-CYSee versus VEH.

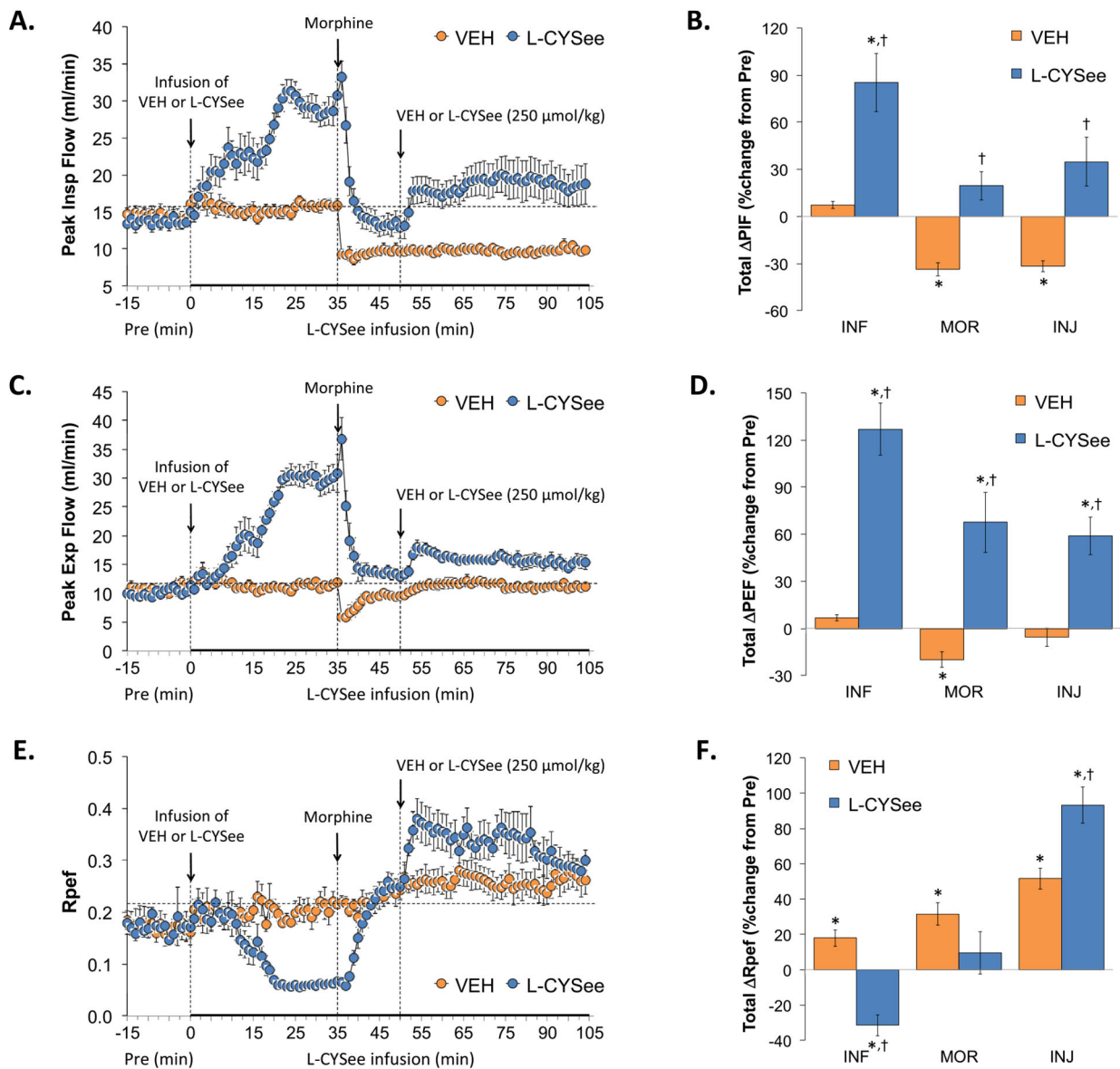


Fig. 4. Changes in peak inspiratory flow (PIF), peak expiratory flow (PEF), and rate of achieving PEF (Rpef). **Panels A, C, and E:** Actual PIF, PEF, and Rpef values during the various stages of the experiment, respectively. **Panels B, D, and F:** Sum of the PIF, PEF, and Rpef responses recorded during the infusion of vehicle (VEH; 20 μL/min, IV) or L-CYSee (INF; 14.3 μmol/kg/min, IV), upon injection of morphine (MOR, 10 mg/kg, IV) and following injection of VEH (100 μL/100 g body weight, IV) or L-CYSee (250 μmol/kg, IV), respectively. Data are presented as mean ± SEM. There were 5 rats in the vehicle-infusion group and 6 rats in the L-CYSee-infusion group. *P < 0.05, significant change from Pre values. †P < 0.05, L-CYSee versus VEH.

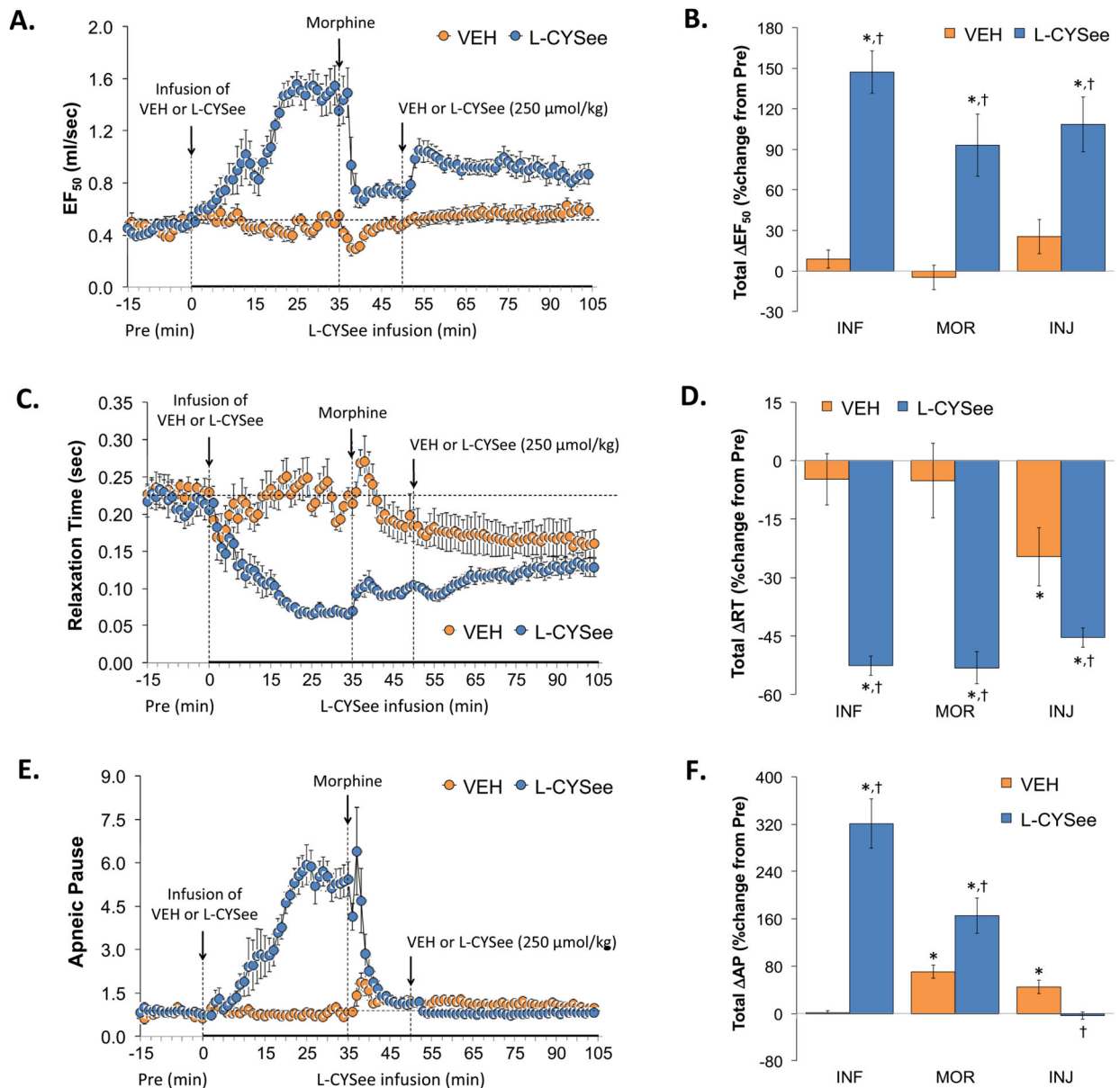


Fig. 5. Changes in expiratory flow at 50% expired tidal volume (EF_{50}), relaxation time (RT), and apneic pause (ApP). **Panels A, C, and E:** Actual EF_{50} , RT, and ApP values during the various stages of the experiment, respectively. **Panels B, D, and F:** Sum of EF_{50} , RT, and ApP responses recorded during the infusion of vehicle (VEH; 20 μ L/min, IV) or L-CYSee (INF; 14.3 μ mol/kg/min, IV), upon injection of morphine (MOR, 10 mg/kg, IV) and following injection of VEH (100 μ L/100 g body weight, IV) or L-CYSee (250 μ mol/kg, IV), respectively. Data are presented as mean \pm SEM. There were 5 rats in the vehicle-infusion group and 6 rats in the L-CYSee-infusion group. * $P < 0.05$, significant change from Pre values. † $P < 0.05$, L-CYSee versus VEH.

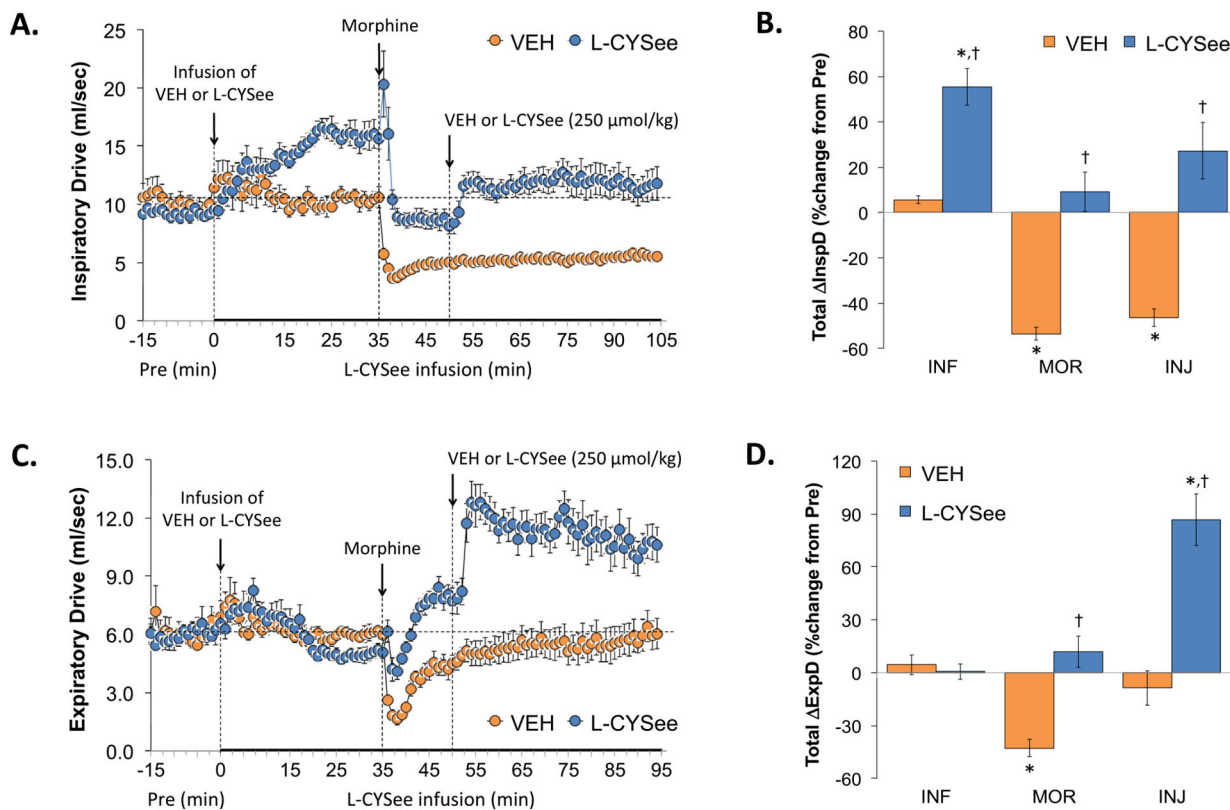


Fig. 6. Changes in inspiratory drive (InspD) and expiratory drive (ExpD). **Panels A and C:** Actual InspD and ExpD values during the various stages of the experiment, respectively. **Panels B and D:** Sum of InspD and ExpD responses recorded during infusion of vehicle (VEH; 20 μL/min, IV) or L-CYSee (INF; 14.3 μmol/kg/min, IV), upon injection of morphine (MOR, 10 mg/kg, IV) and following injection of VEH (100 μL/100 g body weight, IV) or L-CYSee (250 μmol/kg, IV), respectively. Data are presented as mean ± SEM. There were 5 rats in the vehicle-infusion group and 6 rats in the L-CYSee-infusion group. *P < 0.05, significant change from Pre values. †P < 0.05, L-CYSee versus VEH.

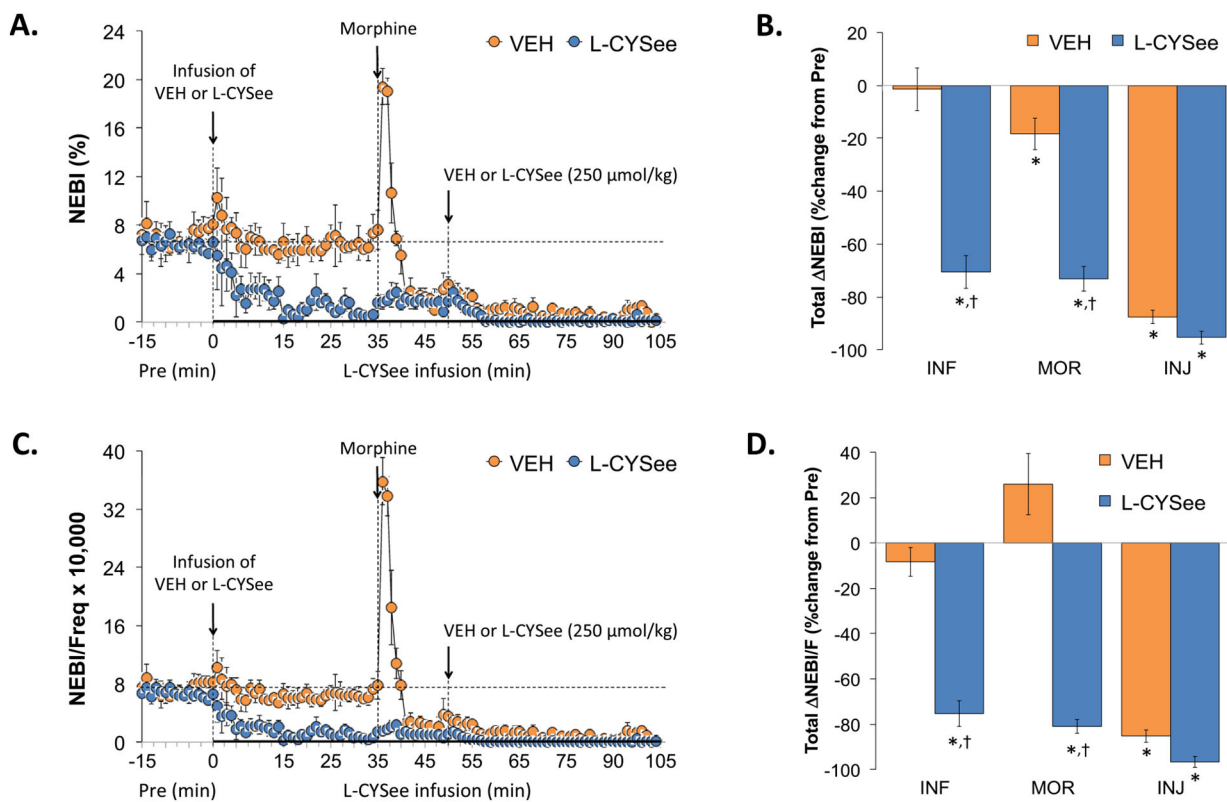


Fig. 7.

Changes in non-eupneic breathing index (NEBI) and NEBI/frequency of breathing (NEBI/Freq). **Panels A and C:** NEBI and NEBI/Freq values during various stages of the experiment, respectively. **Panels B and D:** Sum of NEBI and NEBI/Freq responses recorded during infusion of vehicle (VEH; 20 μL/min, IV) or L-CYSee (INF; 14.3 μmol/kg/min, IV), upon injection of morphine (MOR, 10 mg/kg, IV) and following injection of VEH (100 μL/100 g body weight, IV) or L-CYSee (250 μmol/kg, IV), respectively. Data are presented as mean ± SEM. There were 5 rats in the vehicle-infusion group and 6 rats in the L-CYSee-infusion group. * $P < 0.05$, significant change from Pre values. † $P < 0.05$, L-CYSee versus VEH.

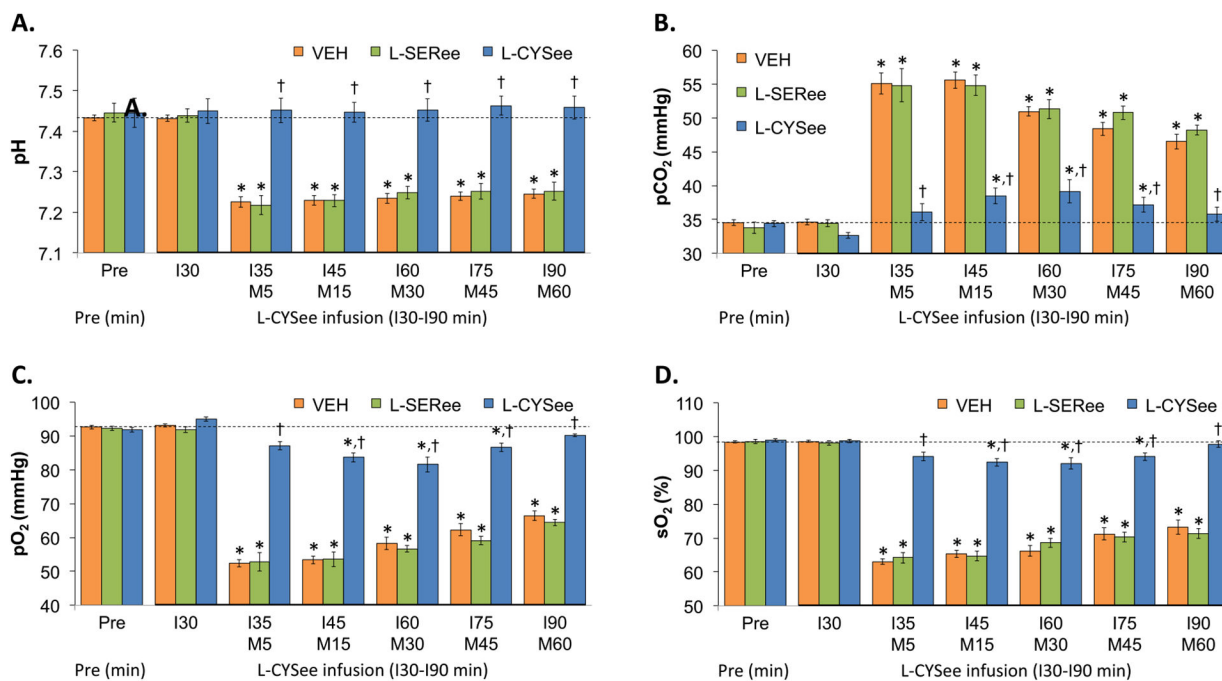


Fig. 8. Actual values for pH (**Panel A**), pCO₂ (**Panel B**), pO₂ (**Panel C**), and sO₂ (**Panel D**) prior to (Pre) following a bolus injection of morphine (M5–M60; 10 mg/kg, IV) in freely-moving rats receiving a continuous infusion (I30–I90) of vehicle (VEH; 10 μL/min), L-serine ethyl ester (L-SERee, 7.15 μmol/kg/min, IV) or L-cysteine ethyl ester (L-CYSee, 7.15 μmol/kg/min, IV). The data are presented as mean ± SEM. There were 8 rats in the vehicle-infusion group and 6 rats in the L-SERee-infusion and L-CYSee-infusion group. *P < 0.05, significant change from Pre values. †P < 0.05, L-CYSee versus VEH.

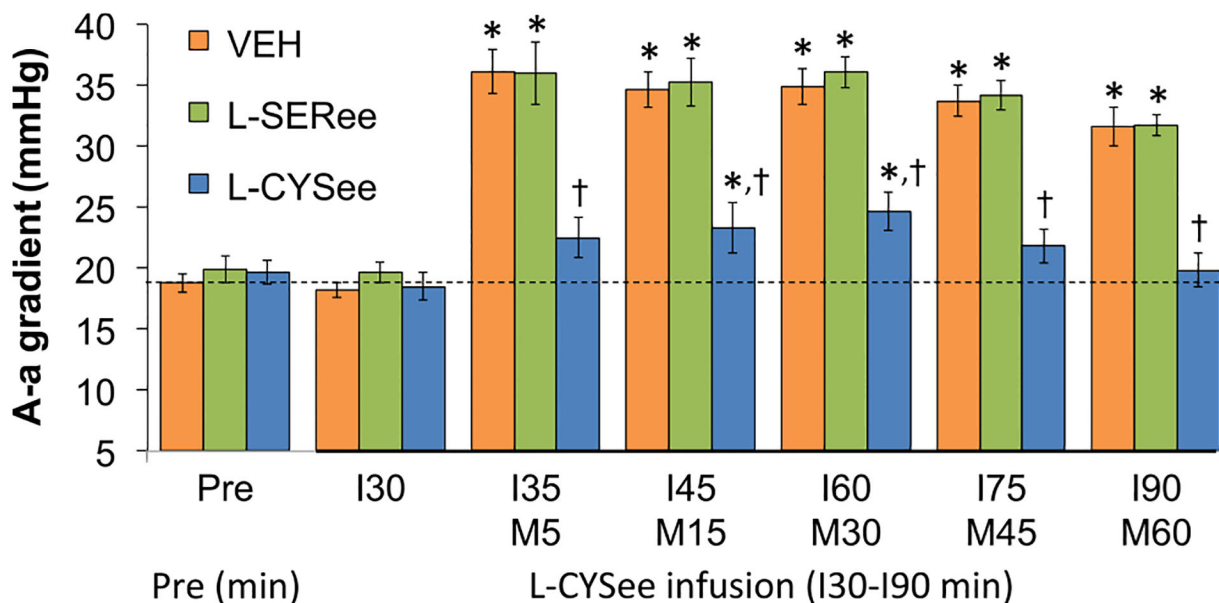
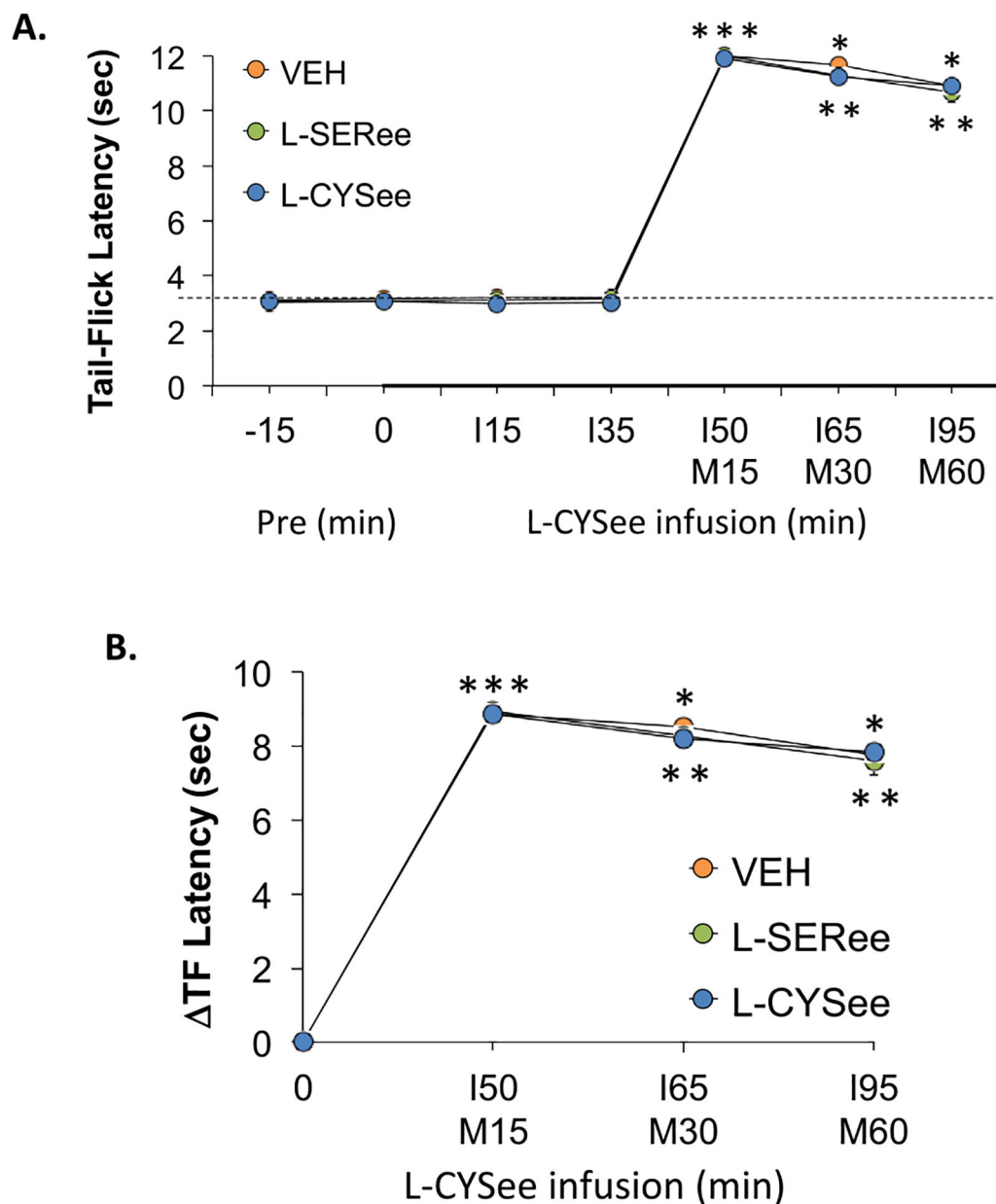


Fig. 9.

Actual values for Alveolar-arterial (A-a) gradient prior to (Pre) following an injection of morphine (M5–M60; 10 mg/kg, IV) in freely-moving rats receiving a continuous infusion (I30–I90) of vehicle (VEH; 10 μ L/min), L-serine ethyl ester (L-SERee, 7.15 μ mol/kg/min, IV) or L-cysteine ethyl ester (L-CYSee, 7.15 μ mol/kg/min, IV). The data are presented as mean \pm SEM. There were 8 rats in the vehicle-infusion group and 6 rats in the L-SERee-infusion and L-CYSee-infusion group. * $P < 0.05$, significant change from Pre values. † $P < 0.05$, L-CYSee versus VEH.

**Fig. 10.**

Panel A: Actual tail-flick (TF) latency values prior to (Pre) following an injection of morphine (M15-M60; 10 mg/kg, IV) in freely-moving rats receiving a continuous infusion (I15-I95) of vehicle (VEH; 20 μ L/min), L-serine ethyl ester (L-SERee, 14.3 μ mol/kg/min, IV) or L-cysteine ethyl ester (L-CYSee, 14.3 μ mol/kg/min, IV). **Panel B:** Arithmetic changes in TF latency. The data are presented as mean \pm SEM. There were 6 rats in each group. * $P < 0.05$, a significant change from Pre values. † $P < 0.05$, L-CYSee versus VEH.

Table 1

Definition of ventilatory parameters.

Parameter	Abbreviation	Units	Definition
Frequency of breathing	Freq	breaths/min	Frequency of breaths per min
Tidal volume	TV	ml	Volume of air per breath
*Minute ventilation	$MV = \text{Freq} \times TV$	ml/min	Total volume of air breathed in per min
Inspiratory time	Ti	sec	Duration of inspiration
Expiratory time	Te	sec	Duration of expiration
End Inspiratory Pause	EIP	msec	Pause between the end of inspiration and start of expiration
End Expiratory Pause	EEP	msec	Pause between the end of expiration and start of inspiration
Relaxation time	RT	sec	Time to expire 64% of tidal volume: non-elastic phase
Peak inspiratory flow	PIF	ml/sec	Peak of inspiratory flow
Peak expiratory flow	PEF	ml/sec	Peak of expiratory flow
EF ₅₀	EF ₅₀	ml/sec	Air-flow at 50% of expired TV
Rp _{ef}	Rp _{ef}	none	Point in expiration when PEF occurs expressed as a fraction of Te
*Apneic Pause	$AP = (Te/RT) - 1$	none	Relative pause in expiration – index of increased airway resistance
*Inspiratory drive	TV/Ti	ml/sec	Drive to inspire air
*Expiratory drive	TV/Te	ml/sec	Drive to expire air
Non-eupneic breathing index	NEBI	% of breaths	Occurrence of non-eupneic breathing events such as apneas
*NEBI/Frequency of breathing	NEBI/Freq	%/(breaths/min)	NEBI adjusted for frequency of breathing

*Parameters denoted with an asterisk were computed from directly recorded parameters

Table 2

Baseline (pre) values in the groups that received vehicle or L-CYSee.

Parameter	Vehicle	L-CYSee
N	5	6
Age, days	79.1 ± 0.3	78.7 ± 0.3
Body weights, grams	335 ± 2	333 ± 2
Frequency, breaths/min	94.8 ± 2.6	97.0 ± 5.2
Tidal Volume (TV), ml	2.40 ± 0.04	2.33 ± 0.09
Minute Ventilation, ml/min	228 ± 9	224 ± 5
Inspiratory Time (Ti), sec	0.24 ± 0.02	0.26 ± 0.01
Expiratory Time (Te), sec	0.41 ± 0.03	0.40 ± 0.03
Inspiratory Time/Expiratory Time	0.61 ± 0.07	0.66 ± 0.04
Ti/(Ti + Te)	37.4 ± 3.0	39.4 ± 1.5
End Inspiratory Pause, msec	8.1 ± 0.3	8.0 ± 0.3
End Expiratory Pause, msec	56.4 ± 4.8	58.8 ± 3.4
Peak Inspiratory Flow, ml/sec	14.6 ± 1.0	13.8 ± 0.6
Peak Expiratory Flow, ml/sec	10.9 ± 0.5	10.1 ± 0.6
Rpef	0.17 ± 0.02	0.17 ± 0.01
EF ₅₀ , ml/sec	0.46 ± 0.03	0.46 ± 0.04
Inspiratory Drive (TV/Ti), ml/sec	10.3 ± 0.9	9.2 ± 0.3
Inspiratory Drive (TV/Te), ml/sec	6.1 ± 0.5	6.0 ± 0.3
Apneic Pause, sec	0.79 ± 0.06	0.86 ± 0.03
Non-Eupneic Breathing Index (NEBI), %	7.0 ± 0.6	6.5 ± 0.2
NEBI/Freq, (%/(breaths/min)) × 100	7.4 ± 0.6	6.7 ± 0.4

L-CYSee, L-cysteine ethyl ester. There were 5 rats in the vehicle group and 6 rats in the L-CYSee group. The data are presented as mean ± SEM. There were no between group differences for any parameter ($P > 0.05$, for all comparisons).