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S-nitroso-L-cysteine stereoselectively blunts the adverse effects of morphine on breathing and arterial blood gas chemistry while promoting analgesia

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Ethics Statement

The Institutional Animal Care And Use Committees (IACUC) of the University Of Virginia (Charlottesville, VA), Case Western Reserve University (Cleveland, OH), and *Galleon Pharmaceuticals, Inc.* (Horsham, PA) provided official approval for the studies presented in this manuscript.

Declaration of conflicting interests

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113436.

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Abstract

S-nitrosothiols exert multiple effects on neural processes in the central and peripheral nervous system. This study shows that intravenous infusion of S-nitroso-L-cysteine (SNO-L-CYS, 1 $\mu\text{mol/kg/min}$) in anesthetized male Sprague Dawley rats elicits (a) sustained increases in minute ventilation, via increases in frequency of breathing and tidal volume, (b) a decrease in Alveolar-arterial (A-a) gradient, thus improving alveolar gas-exchange, (c) concomitant changes in arterial blood-gas chemistry, such as an increase in pO_2 and a decrease in pCO_2 , (d) a decrease in mean arterial blood pressure (MAP), and (e) an increase in tail-flick (TF) latency (antinociception). Infusion of S-nitroso-D-cysteine (SNO-D-CYS, 1 $\mu\text{mol/kg/min}$, IV), did not elicit similar responses, except for a sustained decrease in MAP equivalent to that elicited by SNO-L-CYS. A bolus injection of morphine (2 mg/kg, IV) in rats receiving an infusion of vehicle elicited (a) sustained decreases in frequency of breathing tidal volume, and therefore minute ventilation, (b) a sustained decrease in MAP, (c) sustained decreases in pH, pO_2 and maximal sO_2 with sustained increases in pCO_2 and A-a gradient, and (d) a sustained increase in TF latency. In rats receiving SNO-L-CYS infusion, morphine elicited markedly smaller changes in minute ventilation, arterial blood gas chemistry, A-a gradient and MAP. In contrast, the antinociceptive effects of morphine were enhanced in rats receiving the infusion of SNO-L-CYS. The morphine-induced responses in rats receiving SNO-D-CYS infusion were similar to vehicle-infused rats. These data are the first to demonstrate that infusion of an S-nitrosothiol, such as SNO-L-CYS, can stereoselectively ameliorate the adverse effects of morphine on breathing and alveolar gas exchange while promoting antinociception.

Keywords

S-nitrosothiol; Morphine; Respiratory depression; Antinociception; Sprague Dawley rats

1. Introduction

S-nitrosothiols are endogenous compounds that affect neural processes within the central [1–8] and peripheral [9–14] nervous systems. Extracellular and intracellular S-nitrosothiols affect neural activity via multiple mechanisms, including (a) their decomposition to nitric oxide (NO) and formation of di-nitrosothiol-iron complexes and subsequent activation of soluble guanylate cyclase/protein kinase G (PKG)-dependent processes [15–21], and (b) by transferring NO^+ moieties (i.e., S-nitrosylation) to thiol residues of numerous functional proteins, such as N-methyl-D-aspartate (NMDA) glutamatergic receptors [18,22–31]. Some endogenous S-nitrosothiols, including S-nitroso-L-cysteine (SNO-L-CYS), also exert their effects by direct activation of stereoselective recognition sites (i.e., sites that are minimally activated by SNO-D-CYS), such as sites present on voltage-gated K^+ -channels, in a process that does not require S-nitrosylation of the functional protein [14]. Microinjections of SNO-L-CYS into the nucleus tractus solitarius (NTS) increase minute ventilation (V_E) in freely-moving rats [32] and decrease mean arterial blood pressure in anesthetized rats

[33] by stereospecific mechanisms independent of decomposition to nitric oxide (NO). Similarly, microinjections of SNO-L-CYS, but not SNO-D-CYS, into the fourth ventricle [34] or lateral ventricles [35] elicit pronounced hemodynamic responses in freely-moving rats. In addition, it is evident that systemic injections of SNO-L-CYS dose-dependently increases V_E by activation of stereoselective processes within the carotid bodies of freely-moving rats since these responses were not elicited by SNO-D-CYS and because the SNO-L-CYS responses were markedly diminished in rats with bilateral transection of the carotid sinus nerves [14]. Further studies in mice have also confirmed a role of endogenous S-nitrosothiols in the ventilatory responses that occur during and following hypoxic gas challenges [36,37]. Finally, systemic injections of S-nitrosothiols, such as SNO-L-CYS [38] and S-Nitroso- β,β -dimethyl-L-cysteine [39], exert dose-dependent vasodilation in freely-moving and anesthetized rats by processes independent of nitric oxide generation [14,40–43], but that are dependent upon stereoselective recognition sites, such as those present on ion-channels [14,22,44], whose functional status is regulated by oxidation-reduction reactions [45–49].

The role(s) of nitrosyl factors, such as nitric oxide and S-nitrosothiols, in the pharmacological actions of opioids has received much attention with potential roles for nitrosyl factors being identified in μ -opioid receptor (μ -OR) signaling [50–53], opioid tolerance [54–56], fentanyl pre-conditioning processes [57], opioid effects within the eye [58], opioid-induced catalepsy [59], opioid effects on vascular function [60,61], pain processing [62–67], and inflammatory-immunoregulatory processes [68,69]. There is very limited evidence as to the participation of nitrosyl factors in the ventilatory depressant effects of opioids. Teppema et al. [70], reported that the ventilatory depressant effects of morphine are independent of neuronal nitric oxide synthase (nNOS) in anesthetized cats. Pellegrino et al. [62], provided evidence that the ventilatory depressant responses elicited by fourth ventricular infusions of morphine in awake dogs were diminished by prior injection of the nitric oxide synthase (NOS) inhibitor, L-nitro-arginine (L-NA), whereas injection of L-NA following morphine was ineffective. In contrast, pre- or post-treatment with L-NA augmented the analgesic actions of morphine in these dogs [62]. In addition, recent studies from our laboratory [71], provided evidence that the ventilatory depressant effects of fentanyl, a popular synthetic opioid, were augmented in unanesthetized rats that had received the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). This finding by Seckler et al. [71]. suggests that the major role of nitrosyl factors in the rat is to combat the inhibitory actions of opioids on breathing, although the sites and mechanisms responsible for the actions of nitrosyl factors remain to be determined. Current research has shown that S-nitrosothiols modulate G protein-coupled receptor signaling in a reversible and receptor-specific manner [72–74], although neither SNO-L-CYS or S-nitroso-L-glutathione appeared to directly interact with μ -ORs [74].

On the basis of the above findings, the objectives of this study were to (a) determine the ventilatory depressant effects and changes in blood pressure elicited by injection of morphine in isoflurane-anesthetized rats that were receiving a continuous infusion of (i) SNO-L-CYS, a ventilatory stimulant [14], (ii) L-cysteine, infused to determine the degree to which the S-nitrosothiol is the active compound, or (iii) SNO-D-CYS, infused to establish whether stereoselectivity is an important factor; and (b) determine

the antinociceptive actions of morphine in separate groups of unanesthetized male Sprague Dawley rats that were receiving the same protocol described in (i), (ii) and (iii). The primary finding was that the infusion of SNO-L-CYS, but not SNO-D-CYS, markedly blunted the depressant effects of morphine on frequency of breathing (f_R), tidal volume (V_T) and minute ventilation (V_E) while promoting morphine-induced antinociception. These data suggest that exogenously administered SNO-L-CYS modulates the pharmacological effects of morphine via modulation of the intracellular signaling pathways recruited by μ -ORs rather than by direct effects on μ -ORs.

2. Materials and methods

2.1. Permissions and rats

All animal studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80.23) revised in 1996. The protocols were approved by the Institutional Animal Care and Use Committees of the University of Virginia, Case Western Reserve University and *Galleon Pharmaceuticals, Inc.* Adult male Sprague Dawley rats were obtained from ENVIGO Laboratories (Indianapolis, IN). These rats were housed under standard conditions in the animal resource center (ARC) with free access to food and water. Room temperature (22 °C), humidity (48–50 %) and light-dark cycle (12:12 h) were maintained consistently in the ARC and laboratories where the studies took place.

2.2. Surgical procedures and protocols for cardiorespiratory studies

Adult male Sprague Dawley rats were anesthetized with 2.5 % isoflurane in a mixture of oxygen (O_2) and medical grade room-air (50 % O_2 : 50 % room-air). Upon induction of surgical plane of anesthesia, evaluated by loss of the corneal blink reflex and hindlimb withdrawal, the rats were placed in the supine position on a thermostatically controlled blanket (*Harvard Apparatus*, MA, USA) connected to a rectal thermometer to keep body temperature at 37.4 ± 0.2 °C. The rats continued to breathe 2.5 % isoflurane in medical grade room-air via a nose cone. A neck incision was made allowing for the vertical trachea to be cannulated with PE-90 tubing (*Intramedic; Becton & Dickinson*, Franklin Drive, NJ, USA). The tubing was then connected to a pneu-motachometer flow head (MLT1L, *AD Instruments*, CO) to continuously measure respiratory flow [75]. The respiratory flow waveforms were used to determine f_R from the cycle period, and were integrated to calculate V_T . V_E was calculated by multiplying f_R by V_T . The femoral artery was cannulated with a PE-50 catheter (*Intramedic; Becton & Dickinson*, Franklin Drive, NJ, USA). This tubing was connected to a fluid-filled piezo-resistive pressure transducer (SP844, *AD Instruments*, Inc. Bella Vista, Australia) to permit the continuous monitoring of arterial blood pressure, and withdrawal of arterial blood samples (100 μ L each). Another catheter (PE-50 connected to PE-10, the latter inserted into the vessel) was placed in the ipsilateral femoral vein to give a bolus injection of morphine (2 mg/kg, IV) or vehicle (saline, 100 μ L/100 g, IV). A third catheter (PE-50 connected to PE-10, the latter inserted into the vein) was inserted into the right jugular vein and then connected to an infusion pump (Standard Infuse-Withdraw Pump 11 Pico Plus Elite Programmable Syringe Pump; *Harvard Apparatus*, MA, USA) to allow

for the continuous infusion of vehicle (20 $\mu\text{L}/\text{min}$, IV), SNO-L-CYS (1 $\mu\text{mol}/\text{kg}/\text{min}$, IV), L-cysteine (1 $\mu\text{mol}/\text{kg}/\text{min}$, IV) or SNO-D-CYS (1 $\mu\text{mol}/\text{kg}/\text{min}$, IV).

2.3. Arterial blood gas chemistry and alveolar-arterial (A-a) gradient

At pre-determined times during the experiment, arterial blood samples were taken to determine pH, pCO_2 , pO_2 and maximal sO_2 as described in detail previously [76–78]. To collect arterial blood samples, any residual saline between the rat and the tubing connecting the syringe was removed and 125 μL of arterial blood was collected with a pre-heparinized 1 ml syringe. The syringe was capped and gently rotated for 10 s to mix the heparin (Hann's Pharma, Wilmington, DE) and blood to prevent clotting. The sample was immediately injected into the blood gas analyzer (ABL 800 Flex; Radiometer, Westlake, OH) to determine pH, pCO_2 , pO_2 and maximal sO_2 . The Alveolar-arterial (A-a) gradient (i. e., the established index of alveolar gas exchange) was determined as detailed previously [76–78]. In brief, the A-a gradient was determined by the equation, $\text{PAO}_2 - \text{PaO}_2$, where PAO_2 is the partial pressure of alveolar O_2 and PaO_2 is pO_2 in arterial blood. $\text{PAO}_2 = [\text{FiO}_2 \times (\text{P}_{\text{atm}} - \text{P}_{\text{H}_2\text{O}}) - (\text{PaCO}_2/\text{respiratory quotient})]$, where FiO_2 is the fraction of O_2 in inspired air; P_{atm} is atmospheric pressure; $\text{P}_{\text{H}_2\text{O}}$ is the partial pressure of water in inspired air; PaCO_2 is pCO_2 in arterial blood; and respiratory quotient (RQ) is the ratio of CO_2 eliminated/ O_2 consumed. We took FiO_2 of room-air to be 21 % = 0.21, P_{atm} to be 760 mmHg, $\text{P}_{\text{H}_2\text{O}}$ to be 47 mmHg and took the RQ value of our adult male Sprague Dawley rats to be 0.9 [76–78].

2.4. Antinociception surgeries and protocol

The antinociception assays were performed in adult male Sprague Dawley rats that had received two catheter implants under 2.5 % isoflurane anesthesia 7 days previously. This was done in order to test rats that were as free from surgical discomfort as possible. The catheters were exteriorized to the back of the neck and all wounds were closed as described previously [35,38–40]. One catheter (PE-50 connected to PE-10, the latter inserted into the right jugular vein) allowed for continuous infusion of vehicle (20 $\mu\text{L}/\text{min}$, IV), SNO-L-CYS (1 $\mu\text{mol}/\text{kg}/\text{min}$, IV), L-cysteine (1 $\mu\text{mol}/\text{kg}/\text{min}$, IV) or SNO-D-CYS (1 $\mu\text{mol}/\text{kg}/\text{min}$, IV). The other catheter (PE-50 connected to PE-10, the latter inserted into the left jugular vein) was for the injection of morphine (2 mg/kg, IV). On the day of the experiment, antinociception was determined by the radiant heat tail-flick (TF) assay as detailed previously [76–79]. In brief, prior to administration of morphine, the rats were allowed to freely crawl inside a canvas garden glove and were then lightly restrained in the glove to allow for the thermal withdrawal latencies to be determined. After administration of morphine, placement of the rat within the glove was done by the investigator. The TF testing apparatus consisted of a beam of focused radiant heat provided by a 50 W projector lamp, which was focused on the underside of the tail at 1 of 5 sites 8–10 mm apart. TF latency was measured to the nearest 0.1 s as the time from onset of heating of the tail to withdrawal of the tail from the heat. The intensity of the light beam was set so that the baseline TF latencies were about 2.5 s. A cutoff time of 12 s was set to minimize potential damage to the tail upon repeated application of the radiant heat beam. TF latencies were established before and during the various stages of the experiment. The data are shown as actual TF

latencies (sec) and as “maximum possible effect” (%MPE) using the formula, %MPE = [(post-injection TF latency – baseline TF latency)/(12 – baseline TF latency)] × 100.

2.5. Drugs and preparation of S-nitrosothiol infusions

Saline (vehicle) and morphine sulfate solution (50 mg/ml) were purchased from Hospira Inc. (Lake Forest, IL, USA). Working dilutions of morphine (10 mg/ml) was prepared in saline. L-Cysteine hydrochloride and D-cysteine hydrochloride were from Sigma Chemical (St. Louis, MO). Stock solutions of SNO-L-CYS and SNO-D-CYS were prepared just before use, as described previously [14,35,38], by reacting 1 ml solutions of (a) 50 mM sodium nitrite (containing 100 μ L of 1 N HCl) and (b) 50 mM of L- or D-cysteine, which resulted in a stable 25 mM stock solution of approximately pH = 3 of the respective isomers. The solutions were diluted in alkalized saline to increase the pH of the SNO-L-CYS or SNO-D-CYS solutions to approximately pH = 6.8. The SNO-L-CYS and SNO-D-CYS solutions were infused at 1 μ mol/kg/min, with an infusion rate of 20 μ L/min. For example, infusion of a 10 mM solution (10 nmol/ μ L) at 20 μ L/min to a 200 g rat would result in the infusion of (10 nmol × 20 μ L)/0.2 kg = 1000 nmol/kg/min = 1 μ mol/kg/min. The stock and test solutions of SNO-L-CYS and SNO-D-CYS were routinely examined spectrophotometrically [14,35,38,80] to ensure that the concentrations of the stereoisomers were identical.

2.6. Data analyses

All data are presented as mean \pm SEM and were evaluated using *t*-test (simple comparison between two means) or by one-way ANOVA (simple comparisons between three means) and two-way ANOVA (repeated measures analyses comparing between group values at the stated time points) followed by Bonferroni corrections for multiple comparisons between means using the error mean square terms from each ANOVA analysis [81–83] as detailed previously [84]. A value of $P < 0.05$ was taken as the initial level of statistical significance for the multiple comparisons testing [81–83]. The statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). In accordance with the need for greater transparency of statistical findings [85], we have presented the results of the tests and initial ANOVA analyses underneath each Figure and Table. We present two F-statistics for the repeated measures ANOVA analyses. The first F-statistic presented for the repeated measures ANOVA analyses pertains to between group comparisons. For example, for 3 groups of rats, with 9 rats per group, the F-statistic, F(number of groups - 1, number of rats - 1 for each), would be denoted as $F_{2,24}$. The second F-statistic presented for the repeated measure ANOVA pertains to the interaction over the number of sample times. For instance, 7 repeated measures/timepoints with the F-value designated as sum of n-1 for the 3 groups of 9 rats each = 8 + 8 + 8 = 24, and n-1 rats × n-1 times of observation = 8 × 6 + 8 × 6 + 8 × 6 = 144, thus the F-statistic would be denoted as $F_{24,144}$.

3. Results

3.1. Body weights and baseline values for the treatment groups

The body weights and baseline values for each of the recorded parameters are summarized in Supplemental Tables 1–3. There were no significant between group differences for any body weight or parameter values in these studies ($P > 0.05$, for all comparisons). The

F-statistics from the one-way ANOVAs for three group comparisons and *t*-statistics for the two group comparisons are provided in the legends of Supplemental Tables 1–3.

3.2. Ventilatory parameters

As seen in Fig. 1, the continuous infusion of vehicle did not alter f_R , V_T , V_E or MAP, whereas the infusion of SNO-L-CYS (1 $\mu\text{mol/kg/min}$, IV) elicited sustained increases in f_R , V_T , and therefore V_E that were accompanied by a sustained decrease in MAP. Although the infusion of SNO-D-CYS (1 $\mu\text{mol/kg/min}$, IV) did not affect f_R , V_T or V_E , it did elicit a sustained decrease in MAP. The bolus injection of morphine (2 mg/kg, IV) given 25 min after commencing vehicle infusion (I25) elicited sustained decreases in f_R , V_T , V_E and MAP in vehicle-treated rats. In rats receiving SNO-L-CYS, the injection of morphine elicited smaller decreases in f_R , V_T and V_E than in vehicle-infused rats, whereas MAP substantially increased over time. The injection of morphine (2 mg/kg, IV) elicited sustained decreases in f_R , V_T and V_E in SNO-D-CYS-infused rats that were of similar magnitude to vehicle-infused rats, and no change in MAP. The repeated measures ANOVA analyses showed significant interactions between treatments and time for f_R ($F_{24,144} = 10.21$, $P < 0.0001$), V_T ($F_{24,144} = 10.62$, $P < 0.0001$), V_E ($F_{24,144} = 13.40$, $P < 0.0001$) and MAP ($F_{24,144} = 6.70$, $P < 0.0001$). The arithmetic changes in f_R , V_T , V_E and MAP elicited by morphine in the rats that received the infusions of vehicle, SNO-L-CYS or SNO-D-CYS, are summarized in Fig. 2. The arithmetic changes in f_R , V_T , V_E and MAP were clearly different between the SNO-L-CYS group of rats and their respective vehicle (control) group, whereas the arithmetic responses in the SNO-D-CYS and their respective vehicle (control) group were similar to one another. As such, the arithmetic responses in the SNO-L-CYS group were significantly different from those in the SNO-D-CYS group. The repeated measures ANOVA analyses showed significant interactions between treatments and time for f_R ($F_{24,144} = 5.66$, $P < 0.0001$), V_T ($F_{24,144} = 10.75$, $P < 0.0001$), V_E ($F_{24,144} = 5.55$, $P < 0.0001$), and MAP ($F_{24,144} = 7.24$, $P < 0.0001$).

3.3. Arterial blood-gas chemistry and A-a gradient

As seen in Fig. 3, the infusion of vehicle or L-cysteine (1 $\mu\text{mol/kg/min}$, IV) did not alter pH, $p\text{CO}_2$, $p\text{O}_2$, maximal $s\text{O}_2$ or A-a gradient, whereas the infusion of SNO-L-CYS (1 $\mu\text{mol/kg/min}$, IV) elicited sustained increases in $p\text{O}_2$ accompanied by sustained decreases in $p\text{CO}_2$ and A-a gradient. The bolus injection of morphine (2 mg/kg, IV) given 25 min after commencing vehicle infusion (I25) elicited sustained decreases in pH, $p\text{O}_2$ and maximal $s\text{O}_2$ that were accompanied by sustained increases in $p\text{CO}_2$ and A-a gradient. In rats receiving the infusion of SNO-L-CYS, the injection of morphine elicited smaller decreases in pH, $p\text{O}_2$ and maximal $s\text{O}_2$ compared to vehicle-treated rats, and smaller increases in $p\text{CO}_2$ and A-a gradient than in vehicle-treated rats. The injection of morphine (2 mg/kg, IV) elicited sustained decreases in pH, $p\text{O}_2$ and maximal $s\text{O}_2$ along with sustained increases in $p\text{CO}_2$ and A-a gradient in L-cysteine-infused rats that were of similar magnitude in the vehicle-infused rats. The repeated measures ANOVA analyses showed significant interactions between treatments and time for pH ($F_{12,84} = 4.95$, $P < 0.0001$), $p\text{CO}_2$ ($F_{12,72} = 5.07$, $P < 0.0001$), $p\text{O}_2$, ($F_{12,72} = 5.74$, $P < 0.0001$), Maximal $s\text{O}_2$ ($F_{12,72} = 8.82$, $P < 0.0001$) and A-a gradient ($F_{12,72} = 5.67$, $P < 0.0001$). As seen in Fig. 4, the infusion of vehicle or SNO-D-CYS (1 $\mu\text{mol/kg/min}$, IV) did not alter pH, $p\text{CO}_2$, $p\text{O}_2$, maximal

sO₂ or A-a gradient. The bolus injection of morphine (2 mg/kg, IV) given 25 min after starting the infusions (I25) elicited sustained decreases in pH, pO₂ and maximal sO₂ that were accompanied by sustained increases in pCO₂ and A-a gradient. These morphine responses were similar in rats receiving infusion of vehicle or SNO-D-CYS. Repeated measures ANOVA analyses showed significant interactions between treatments and time for pH ($F_{8,48} = 20.93$, $P < 0.0001$), pCO₂ ($F_{8,48} = 6.69$, $P < 0.0001$), pO₂ ($F_{8,48} = 10.08$, $P < 0.0001$), maximal sO₂ ($F_{8,48} = 6.92$, $P < 0.0001$) and A-a gradient ($F_{8,48} = 4.33$, $P = 0.0006$). The arithmetic changes in pH, pCO₂, pO₂, maximal sO₂ or A-a gradient elicited by morphine in rats that received the infusions of vehicle, SNO-L-CYS or SNO-D-CYS are summarized in Fig. 5. Arithmetic changes in these parameters elicited by morphine were similar in the vehicle (control) groups for both SNO-L-CYS and SNO-D-CYS. In contrast, the arithmetic responses elicited by morphine were clearly blunted in rats that were receiving SNO-L-CYS infusion in comparison to their vehicle-infused counterparts, whereas the morphine responses were similar in the SNO-D-CYS rats compared to their respective vehicle controls. Accordingly, the arithmetic responses in the SNO-L-CYS group were different from those in the SNO-D-CYS group. The repeated measures ANOVA analyses showed significant interactions between treatments and time for pH ($F_{20,100} = 9.88$, $P < 0.0001$), pCO₂ ($F_{20,100} = 8.05$, $P < 0.0001$), pO₂ ($F_{20,100} = 4.33$, $P < 0.0001$), maximal sO₂ ($F_{20,100} = 6.99$, $P < 0.0001$) and A-a gradient ($F_{20,100} = 4.84$, $P < 0.0001$).

3.4. Antinociception

As shown in Fig. 6, the infusion (1 µmol/kg/min, IV) of SNO-L-CYS, but not SNO-D-CYS, elicited a minor, however sustained increase in tail-flick (TF) latency. The subsequent injection of morphine (2 mg/kg, IV) elicited pronounced and sustained increases in TF latency of rats receiving the infusions of vehicle for both the SNO-L-CYS and SNO-D-CYS groups. The morphine-induced increases in TF latency were more pronounced in the rats that were receiving the infusion of SNO-L-CYS, whereas the morphine-induced responses in the rats receiving SNO-D-CYS were equivalent to their vehicle (control) group. The repeated measures ANOVA analyses showed significant interactions between treatments and time for the SNO-L-CYS study (Panel A, $F_{21,126} = 2.03$, $P = 0.0088$) and SNO-D-CYS study (Panel B, $F_{16, 96} = 3.105$, $P = 0.0003$). Repeated measures ANOVA statistics for arithmetic changes in TF latencies (including the L-Cysteine group not shown in panels) shown in Panel C also showed significant interactions between treatments and time ($F_{37,185} = 3.27$, $P < 0.0001$). These findings expressed as a percentage of maximum possible effect (%MPE) are summarized in Supplemental Table 4. Morphine-induced increases in TF latency were greater in rats receiving infusion of SNO-L-CYS, whereas the antinociceptive effects of morphine were not altered in rats that were receiving infusions (1 µmol/kg/min, IV) of L-cysteine or SNO-D-CYS. The repeated measures ANOVA analyses showed significant interactions between treatments and time for the SNO-L-CYS study ($F_{21,105} = 2.37$, $P = 0.0022$) and the SNO-D-CYS study ($F_{16,80} = 4.14$, $P < 0.0001$).

4. Discussion

In this study SNO-L-CYS blunts the adverse effects of morphine on breathing without reducing the analgesic effects of the opioid. SNO-L-CYS is produced primarily by

metalloproteins, such as nitric oxide synthase (NOS), ceruloplasmin and hemoglobin [26,27,86,87–95]. It is well-known that S-nitrosothiols are important extracellular and intracellular signaling molecules [20,26,27]. For example, the hemoglobin R to T conformational switch caused by acidosis, hypercapnia, hypoxia and other physiological phenomena, stimulates the formation of signaling S-nitrosothiols, such as SNO-L-CYS [14,20,27,86–92]. Formation of S-nitrosothiols by NOS in endothelial cells and erythrocytes is important for blood pressure regulation, but not for regulation of peripheral blood flow. However, the opposite is true for SNO-L-CYS and other S-nitrosothiols formed during hemoglobin deoxygenation [89,93, 95]. Recent evidence suggests that this class of low mass thiol-NO adducts is also stored in endothelial and neuronal vesicles for subsequent release [96]. Abnormal S-nitrosothiol levels have been measured *ex vivo* in a variety of human conditions, ranging from altitude acclimatization to diseases like asthma, pulmonary hypertension, and sepsis [89,90,97, 98,99].

4.1. Effects of SNO-CYS and SNO-D-CYS on baseline parameters

The present study demonstrates that the continuous intravenous infusion (1 $\mu\text{mol/kg/min}$) of the endogenous S-nitrosothiol, SNO-L-CYS [80,96,100], elicits increases in f_R , V_T and V_E in isoflurane-anesthetized rats. The lack of effects of the L-cysteine infusion suggests that the nitrosylation of the thiol residue in cysteine allows SNO-L-CYS activity that is not possessed by the parent thiol. The finding that SNO-D-CYS elicited minimal changes in ventilatory parameters supports current evidence that the ability of SNO-L-CYS to increase V_E upon microinjection into the NTS [32] or upon systemic delivery to carotid bodies [14] involves stereoselective processes. These processes may include the ability of SNO-L-CYS, but not SNO-D-CYS, to undergo intracellular transport via an L-amino acid transporter system [101,102] to allow SNO-L-CYS to modulate the function/activities of intracellular signaling proteins [7,72,103–111]. These stereoselective processes may also include interaction with membrane-bound proteins, such as N-methyl-D-aspartate glutamatergic ligand-gated ion-channel receptors [44], T-type Ca^{2+} -channels [22], and voltage-gated K^+ -channel sub-type 1.1 ($\text{Kv}_{1.1}$ -channels) [14]. Additionally, Ca^{2+} -activated K^+ -channels [18,23,105,112,113] and $\text{Kv}_{1.2}$ - and $\text{Kv}_{1.3}$ -channels [114], although stereoselectivity of the effect has not been confirmed for these channels.

The present study also shows that the continuous infusions of SNO-L-CYS and SNO-D-CYS at 1 $\mu\text{mol/kg/min}$ elicited equivalent and substantial reductions in MAP in the isoflurane-anesthetized rats. We have reported that bolus intravenous injections of S-nitrosothiols, such as SNO-L-CYS [38] and S-nitroso- β,β -dimethyl-L-cysteine [39], exert dose-dependent reductions in MAP due to reductions in regional vascular resistances in freely-moving and anesthetized rats [14,38–41]. The potencies of the respective D-isomers at lower doses (e.g., 10–200 nmol/kg, IV) are much smaller than that of the L-isomers in freely-moving rats, but the differences in potency diminish when higher doses (e.g., 400–800 nmol/kg, IV) of L- and D-isomers are given [38,39]. Moreover, the difference in potency of L- and D-isomers is smaller at all doses in anesthetized rats compared to freely-moving rats [39]. Thus, the similar reductions in MAP elicited by the infusion of SNO-L-CYS and SNO-D-CYS in the anesthetized rats is consistent with evidence that although infusion of SNO-L-CYS into the femoral artery of anesthetized sheep to elicit

blood concentrations of approximately 0.6 $\mu\text{mol/L}$ induced a pronounced vasodilation in the ipsilateral hindlimb, infusion of these concentrations of SNO-D-CYS did not. Nonetheless, both infusions caused pronounced decreases in MAP upon reaching the general circulation. At present, the processes by which anesthetics alter the relative potencies of the L- and D-isomers remain to be determined [39]. Previous data suggest the equivalent decreases in MAP elicited by the infusions of SNO-L-CYS and SNO-D-CYS may involve the trans-nitrosylation of circulating proteins, such as albumin [115], to form the vasodilator, S-nitroso-albumin [116,117]. The infusion of SNO-L-CYS increased TF latency in freely-moving rats, whereas similar infusions L-cysteine or SNO-D-CYS did not. The rats showed no overt behavioral responses to the infusion of the SNO-L-CYS. In other words, there were instances of grooming events and movement during the infusion period and these events were not obviously different in frequency to the pre-infusion period or to those of the other infusion groups (e.g., L-cysteine and SNO-D-CYS). As such it is tempting to assume that the increase in TF latency was an actual antinociception effect by mechanisms and sites of action that we have not established at present. However, it has been established that SNO-L-CYS exerts pronounced antinociception by stereoselective effects via L-amino acid transport mechanisms in rat spinal cord [118–120], and potentially by stereoselective inhibition of $\text{CaV}_{3,2}$ T-type Ca^{2+} -channels within the thalamus and dorsal root ganglia [12,22].

4.2. Effects of SNO-L-CYS on morphine-induced responses

Key findings of this study were that the depressant effects of morphine on ventilatory parameters, ABG chemistry and A-a gradient were substantially *diminished* in Sprague Dawley rats that were receiving a continuous infusion of SNO-L-CYS, whereas the antinociceptive effects of the morphine were *augmented* during the infusion of SNO-L-CYS. The finding that morphine decreased MAP in vehicle-infused rats, whereas it reversed the hypotension elicited by SNO-L-CYS infusion, but not SNO-D-CYS infusion, also speaks to a complicated, but important interaction, between the signaling mechanisms elicited by SNO-L-CYS and morphine. Although far from definitive, these findings are consistent with evidence that S-nitrosothiols do not directly interact with $\mu\text{-ORs}$ [74], and suggest that SNO-L-CYS modulates the mechanisms and signaling pathways that are proposed to mediate the pharmacological actions of morphine [77,121–123]. The precise mechanisms by which SNO-L-CYS exerts these effects have yet to be established, however, the lack of efficacy of L-cysteine and SNO-D-CYS certainly suggests that stereoselective mechanisms, including intracellular uptake via an L-amino acid transporter system [101,102], thereby allowing SNO-L-CYS to modulate numerous intracellular signaling cascades involved in the cardiorespiratory system [72,103–111], are involved. We have reported that the NADPH diaphorase histochemical technique identifies the presence of S-nitrosylated proteins in central and peripheral tissues [96]. Evidence that acute administration of morphine elicits a marked decrease in NADPH diaphorase staining within the brain [124–126], raises the intriguing possibility that morphine promotes the use and/or de-nitrosylation of S-nitrosylated species within the brain, and possibly the peripheral structures that are normally rich in NADPH diaphorase staining, including the carotid bodies [127]. Moreover, prevention of the potential depletion of endogenous S-nitrosylated proteins (e.g., L-SNO-proteins) by the infusion of SNO-L-CYS may play a key role in (i) diminishing the adverse

cardiorespiratory actions of morphine (i.e., those on ventilatory parameters, ABG chemistry and A-a gradient) while (ii) enhancing the antinociceptive actions of the opioid.

4.3. Effects of SNO-L-CYS on morphine-induced changes in ventilatory parameters

In agreement with an earlier study in isoflurane-anesthetized rats [128], the bolus intravenous injection of a 2 mg/kg dose of morphine elicited sustained decreases in arterial blood pH, pO₂ and sO₂ that were accompanied by an increase in pCO₂, with all responses being indicative of hypoventilation, and pronounced increases in Alveolar-arterial gradient, which is indicative of ventilation-perfusion mismatch in alveoli. These responses closely align with a general understanding of the pharmacological effects of morphine in rats and humans, including those under anesthesia [128–131]. The ability of morphine to depress the ventilatory responses to hypoxic [132–134] and hypercapnic [134] gas challenges in humans, suggests that morphine and/or its metabolites [135] may depress central and peripheral (e.g., the carotid body) mechanisms that respond to these challenges [133,134]. It is established that morphine blunts ventilatory responses to hypoxic or hypercapnic gas challenges in rats [136–139]. Morphine probably blunts these ventilatory responses by actions in the central nervous system because (i) μ -ORs are expressed in nuclei involved in ventilatory control processes [140], (ii) microinjections of the μ -OR agonist, DAMGO, into the commissural NTS suppresses the ventilatory responses to hypoxic or hypercapnic gas challenges [140–142], and (iii) microinjections of DAMGO into the medullary raphe region of rats attenuate responses to hypoxic gas challenge. The probability that morphine suppresses ventilatory responses to hypoxic or hypercapnic challenges via peripheral actions was addressed by McQueen and Ribeiro [143] in anesthetized cats by examining the effects of intra-carotid administration of morphine or the δ -OR agonist, methionine-enkephalin, on chemoreceptor fiber activity in the carotid sinus nerve (CSN). Unexpectedly, they found that (i) morphine was minimally inhibitory at higher doses, while lower doses increased CSN discharge, and (ii) methionine-enkephalin elicited profound inhibition of CSN discharge [143]. In addition, CSN firing discharge evoked by intra-carotid injections of CO₂-saturated buffer solutions were *augmented* by morphine, whereas they were reduced by methionine-enkephalin [143]. In a later study, Kirby and McQueen [144] found that opioid depression of chemosensory discharge in cats involved δ -ORs rather than μ -ORs. It should also be noted that Zimpfer et al. [145] reported that morphine markedly attenuated the cardiovascular responses due to chemoafferent stimulation in conscious dogs. Whether this effect of morphine was due to actions in the brain and/or carotid bodies was not established.

Surprisingly, the possibility that morphine affects carotid body and CSN discharge or the responses of these structures to hypoxic and/or hypercapnic challenges, have not been examined directly in rats. We examined this possibility indirectly by determining the adverse effects of morphine (10 mg/kg, IV) on ventilatory responses elicited by hypoxic or hypercapnic challenges in freely-moving Sprague Dawley rats with prior (7 day) sham-operation or those with bilateral CSN transection, which disrupts chemosensory afferent input to the NTS [146]. We found that the ability of morphine to suppress ventilatory responses to hypoxic or hypercapnic challenges were actually *exacerbated* in CSN transected rats, providing the first in vivo evidence that carotid body chemoreceptors defend against, rather than participate in, the ability of morphine to depress breathing in

rats. These findings align with those of McQueen and Ribeiro [143], and suggest that the ability of SNO-L-CYS infusion to prevent morphine-induced suppression of breathing somehow involves the actions of this S-nitrosothiol within the carotid bodies. Recently, evidence has shown that SNO-L-CYS drives breathing via actions within the carotid bodies, and thus it is apparent that the L-isomer can affect signaling processes within the carotid body chemosensitive glomus cells and/or chemoafferent nerve terminals [14]. As such, the infusion of SNO-L-CYS may in some way allow morphine-induced stimulation of μ -ORs to promote glomus cell and/or chemoafferent activity thereby counteracting the adverse effects of morphine on breathing via other mechanisms (see below). Low doses of morphine (e.g., 1 mg/kg, IV) increase ventilation in adult [147] and fetal [148,149] rats. In adult rats, excitatory ventilatory responses elicited by morphine include increases in (i) f_R (accompanied by decreases in inspiratory time and end inspiratory pause), (ii) V_E , (iii) inspiratory drive, and (iv) peak inspiratory and expiratory flows [147]. Evidence has shown that the ability of the low dose of morphine to drive breathing involved activation of central μ -and/or δ -ORs, and that the ability of morphine to depress breathing by activation of peripheral μ -and/or δ -ORs masks the central actions. This evidence complements that of Szeto et al. [148], who found that morphine-induced stimulation of fetal breathing involved stimulation of μ_1 -ORs and activation of central muscarinic pathways. Whether infusion of SNO-L-CYS enhances the activity of the above mentioned central systems is not known, but it is known that central administration of SNO-L-CYS elicits an array of cardiorespiratory responses [32–35].

4.4. Effects of SNO-L-CYS on morphine-induced changes in A-a gradient

The precise mechanisms by which morphine increases A-a gradient, which compromises gas-exchange in lung alveoli, in naïve or isoflurane-anesthetized rats are unknown and a comprehensive search of the literature found no relevant publications. On the basis of numerous studies in humans, the two leading mechanisms by which morphine and isoflurane could increase A-a gradient would be via (i) atelectasis, which is collapse of alveoli via changes in intra-pleural pressures due to hypoventilation and/or changes in the effective concentration of surfactants responsible for maintaining alveolar integrity, and (ii) impaired clearance of fluids from the alveoli rather than by changes in pulmonary artery or pulmonary wedge pressures [150–152]. Indeed, isoflurane [153] and opioids, such as morphine [154–156] and fentanyl/fentanyl analogs [157,158], cause/promote atelectasis in humans, whereas there is little information as to whether opioids adversely affect fluid clearance from alveoli [159,160]. Although there is a lack of evidence with opioids, there is direct evidence that isoflurane decreases alveolar epithelial fluid clearance in rats [161] although the A-a gradients recorded in our isoflurane-anesthetized rats of the present study were very similar to those recorded in freely-moving unanesthetized rats [76–78]. It is possible that the interaction of isoflurane and morphine leads to a more dramatic effect of morphine on intracellular processes that underlie the increases in A-a gradient elicited by the opioid. Whether the mechanisms by which morphine inhibits gas transfer within the lungs involve atelectasis, diminished fluid clearance in alveoli, or other potential mechanisms [150–152], an obvious question relates to how SNO-L-CYS can markedly diminish the increase in A-a gradient elicited by morphine. The decomposition of SNO-L-CYS is unlikely to be involved since nitric oxide inhibits alveolar fluid clearance [162], lung sodium transport

through alveolar type 1 and 2 cells [163, 164], and amiloride-sensitive Na^+ transport [165] via soluble guanylate cyclase/cGMP-dependent processes. As such, SNO-L-CYS may prevent the adverse effects of morphine on A-a gradient by direct activation of ion-channels and/or by S-nitrosylation of functional proteins [14] relevant to the inhibitory effects of morphine. To date there is no available data regarding whether, for example, SNO-L-CYS is able to directly bind to or indirectly modulate, via S-nitrosylation, the amiloride sensitive Na^+ transport system. We are currently performing studies using elegant techniques developed by others [166–168], to obtain evidence as to how morphine and fentanyl diminish the clearance of fluid from alveoli and reduce amiloride-sensitive transepithelial Na^+ transport, and the mechanism by which SNO-L-CYS modulates these effects.

4.5. Effects of SNO-L-CYS on morphine-induced changes in MAP

As expected, the injection of morphine elicited a sustained decrease in MAP in the vehicle-treated rats. This is consistent with reports that systemic administration of morphine elicits complex dose-dependent effects on hemodynamic parameters and baroreceptor reflex activity in both humans and animals, including rats, where hypotension is a commonly reported phenomenon [75,169–175]. A complicated finding was that morphine caused a sustained reversal of the hypotension in rats receiving the infusion of SNO-L-CYS. We have no direct knowledge of the mechanisms by which this response occurs. Parra et al. [176]. reported that morphine had minimal effects on the contractile status of resting (untreated) aortic strips isolated from rats. However, when the vasoconstrictor effects of the sympathetic neurotransmitter, norepinephrine, were allowed to wane, a process that most likely involves the release of endothelium-derived relaxing factors [177–180], the application of morphine elicited sustained concentration- and μ -OR-dependent vasoconstrictor responses by mechanisms that are yet to be determined. As such, it is possible that infusion of the endogenous endothelium-derived relaxing factor, SNO-L-CYS [80,96,100], to the isoflurane-anesthetized rats described in this study alters μ -OR-dependent signaling processes in vascular smooth muscle, such that the subsequent injection of morphine elicits vasoconstrictor responses, thereby producing the observed time-dependent elevation of MAP compared to prior SNO-L-CYS-infusion levels.

4.6. Effects of SNO-L-CYS on morphine-induced changes in antinociception

There is now considerable evidence that nitrosyl factors play vital roles in the antinociceptive actions of morphine [62–67,181,182]. A variety of elegant studies in rats and mice have delineated some of these roles [130,181], and have shown that morphine antinociception is lost in neuronal NOS knock-out mice [181] and that this antinociception involves the initial stimulation of phosphatidylinositol-4,5-bisphosphate 3-kinase γ ($\text{PI}_3\text{K}\gamma$)-protein kinase B (PKB/Alt) pathway that leads to activation of neuronal NOS and ultimately the increased activation of ATP-dependent K^+ -channels (K_{ATP} -channels) [182]. Our finding that the antinociceptive potency of morphine was enhanced in rats receiving SNO-L-CYS infusion, but not those receiving infusions of L-cysteine and SNO-D-CYS, points to stereoselectivity for SNO-L-CYS. Whether the effects of SNO-L-CYS involve actions in the above pathway are not known, although Numajiri et al. [183]. found that morphine signaling via $\text{PI}_3\text{K}\gamma$ -PKB/Alt involves S-nitrosylation of PKB and a phosphatase with high sequence homology to tensin (PTEN), which regulates $\text{PI}_3\text{K}\gamma$ signaling. An extensive

review of the literature found that while nitrosyl factors exert numerous effects that involve changes in K_{ATP} -channel activity [184], there is no evidence for direct S-nitrosylation of this ion-channel. With respect to disease processes that may involve S-nitrosothiols, it is known that the analgesic potency of opioids is enhanced upon development of sepsis [185,186], a pathological condition which is associated with greatly enhanced levels of circulating S-nitrosothiols [187].

4.7. Study limitations and conclusions

With respect to the study limitations, the findings that the ability of morphine to adversely affect ventilatory parameters, A-a gradient (i.e., the index of gas exchange in alveoli), and arterial blood gas chemistry (i.e., pH, pO_2 , pO_2 , sO_2) is markedly reduced in isoflurane-anesthetized adult male Sprague Dawley rats that were receiving an intravenous infusion of SNO-L-CYS (1 $\mu\text{mol/kg/min}$, IV), needs to be confirmed in freely-moving rats that are unanesthetized. Moreover, the potency and efficacy of SNO-L-CYS needs to be established in dose-response studies to establish whether lower infusion concentrations of SNO-L-CYS are efficacious. Another important limitation of this study is the lack of evidence as to the efficacy of SNO-L-CYS in naïve female rats and female rats given morphine with the knowledge that females often display qualitatively and quantitatively different responses to opioids than males [133,188]. Yet another major limitation in this study is the lack of understanding of the molecular mechanisms by which SNO-L-CYS modulates the pharmacological actions of morphine. Currently we are studying the degree to which the systemic administration of morphine generates S-nitrosothiols in central (e.g., the NTS) and peripheral (e.g., the carotid body) structures in rats using our capacitive sensor technology [189], and whether systemic administration of morphine alters the S-nitrosylation status of central (e.g., the NTS) and peripheral (e.g., the carotid body) structures using NADPH diaphorase histochemistry [96].

In conclusion, this study demonstrates that the adverse effects of morphine on ventilation, ABG chemistry, and A-a gradient were markedly reduced in isoflurane-anesthetized male Sprague Dawley rats that were receiving an intravenous infusion of SNO-L-CYS, but not L-cysteine or SNO-D-CYS. Moreover, we also found that the antinociceptive effects of morphine were augmented in rats receiving the SNO-L-CYS infusion, but not infusions of L-cysteine or SNO-D-CYS. The ability of SNO-L-CYS to exert these effects may involve (i) stereoselective processes, including the activation of membrane signaling proteins [14,22,44,190,191], and/or (ii) intracellular entry via an L-amino acid transporter system, which then allows modulation of intracellular signaling pathways [101, 102]. Based on findings that peripherally restricted μ -OR antagonists (e.g., naloxone methiodide) markedly affect the cardiorespiratory and antinociceptive actions of opioids [76], we also conclude that the ability of SNO-L-CYS to modulate the effects of morphine involves the interaction with both peripheral and central μ -OR signaling pathways. For instance, the ability of morphine to reverse SNO-L-CYS-induced hypotension suggests a remarkable and unexpected interaction between S-nitrosothiol- and μ -OR-signaling pathways. Our findings provide the first direct evidence that circulating SNO-L-CYS attenuates the adverse actions of morphine on breathing, A-a gradient (i.e., gas exchange in the lungs), and arterial blood-gas chemistry in anesthetized rats. Another key finding of this study was that the

antinociceptive actions of morphine were enhanced in freely-moving rats receiving the infusion of SNO-L-CYS. Additionally, the findings that infusions of L-cysteine or SNO-D-CYS were not able to replicate the above actions of SNO-L-CYS strongly suggest that the L-S-nitrosothiol moiety is the active molecule, and that it acts via stereoselective mechanisms. Conclusively, our findings suggest that the presence/bioavailability of SNO-L-CYS, and perhaps the S-nitrosylation status of proteins in general, may play a fundamental role in the expression of the pharmacological effects of morphine and presumably other opioids, such as fentanyl [71]. As such, conditions and disease states that favor the formation or degradation of S-nitrosothiols [2,18,23,31,105,108,117,187] may be significant for determining the efficacy of the pharmacological actions of opioids. The effects of morphine on the different physiological processes examined in this study (ventilation, alveolar gas exchange and antinociception) may have different mechanisms based on differential expression patterns of opioid receptors, μ -ORs, δ -ORs and κ -ORs, within the brain [192–198] and periphery [199–201]. The μ -OR, which is the major receptor for morphine [129–134]), is widely expressed in central [192,193,196,197] and peripheral [199–201] nervous systems. Within the central nervous system, μ -ORs are expressed in different neuron types (excitatory and inhibitory) and in astroglia [192,193,196,197]. The differential expression of μ -ORs in excitatory and inhibitory neurons may play important roles in the effects of morphine on the different physiological processes under investigation in the present study, and therefore may be a major player in how SNO-L-CYS modulated the effects of morphine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interest Statement

The authors declare that this study received funding from *Galleon Pharmaceuticals, Inc. and that Santhosh M. Baby was employed by the company Galleon Pharmaceuticals, Inc.* The leadership of the company was not directly involved in this study as a commercial entity. Only the principal scientists of the company were involved in the study design, collection, analysis, interpretation of data, the writing of this article, and the decision to submit it for publication. The remaining authors declare that the research described in this manuscript was performed in the complete absence of commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The corresponding author will provide the datasets generated from this study upon email request to sjl78@case.edu.

Abbreviations:

A-a gradient	Alveolar-arterial gradient
ABG	arterial blood-gas chemistry
DAMGO	D-Ala ² , N-MePhe ⁴ , Gly-ol]-enkephalin
δ-OR	δ-opioid receptor
fr	frequency of breathing
K_{ATP}-channels	ATP-dependent K ⁺ -channels
L-NA	L-nitro-arginine
L-NAME	N ^G -nitro-L-arginine methyl ester
MAP	mean arterial blood pressure
Ve	minute ventilation
μ-OR	μ-opioid receptor
NO	nitric oxide
NTS	nucleus tractus solitarius
PI₃Kγ	phosphatidylinositol-4,5-bisphosphate 3-kinase γ
PKB/Akt	protein kinase B
SNO-D-CYS	S-nitroso-D-cysteine
SNO-L-CYS	S-nitroso-L-cysteine
TF latency	tail-flick withdrawal latency
Vt	tidal volume

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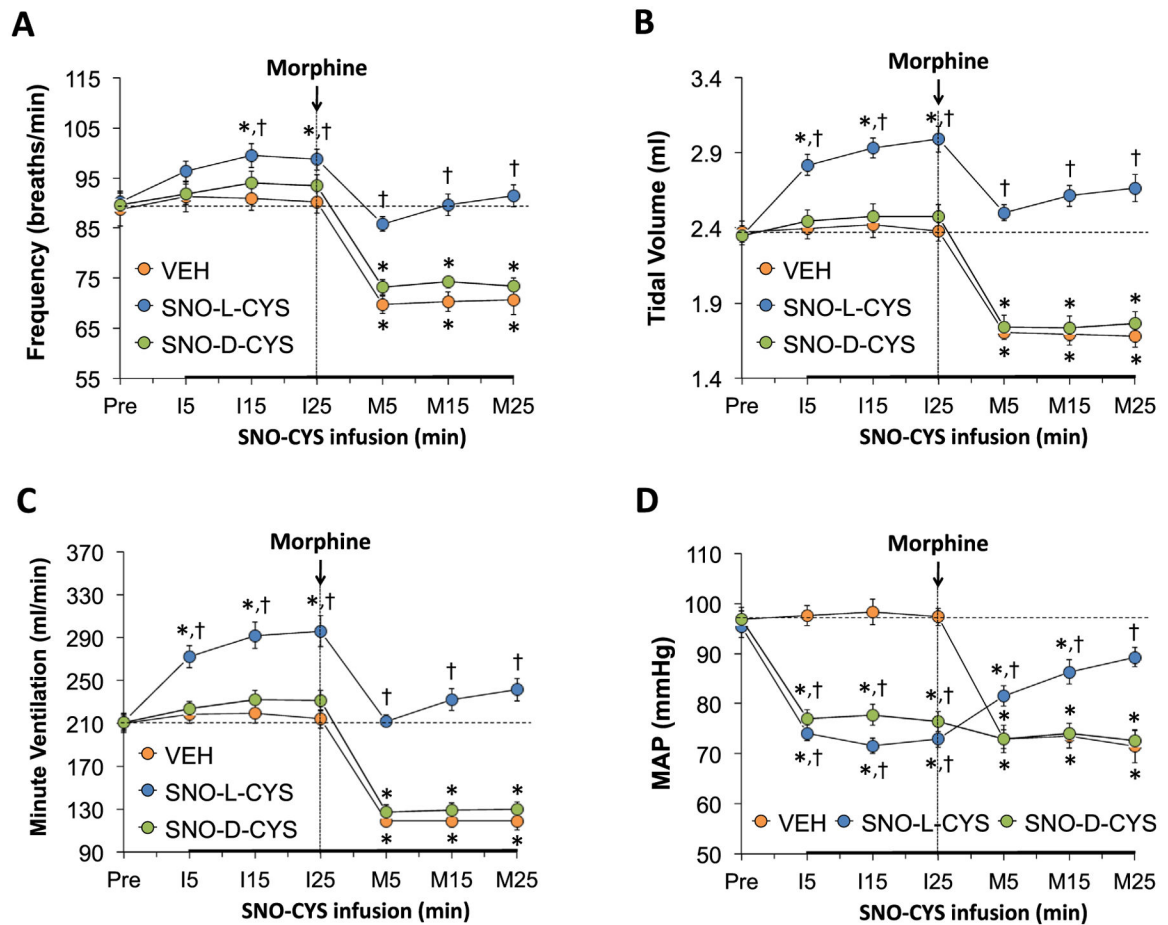


Fig. 1. (Panel A) Frequency of breathing, (Panel B) tidal volume, (Panel C) minute ventilation and (Panel D) mean arterial blood pressure (MAP) values before (Pre), and during the continuous infusion (15 min onward) of vehicle (VEH, 20 μ L/min, IV) or S-nitroso-L-cysteine (SNO-L-CYS, 1 μ mol/kg/min, IV), or S-nitroso-D-cysteine (SNO-D-CYS, 1 μ mol/kg/min, IV) and upon bolus injection of morphine (2 mg/kg, IV; M5 onward) in isoflurane-anesthetized rats. There were 9 rats in each group. Data are presented as mean \pm SEM. * $P < 0.05$, I15, I15 and I25 versus Pre and M5, M15, M25 versus I25. † $P < 0.05$, SNO-L-CYS or SNO-D-CYS versus vehicle. Repeated measures ANOVA statistics: Frequency of breathing, $F_{2,24} = 11.11$, $P = 0.004$, $F_{24,144} = 10.21$, $P < 0.0001$; Tidal volume, $F_{2,24} = 36.01$, $P < 0.0001$, $F_{24,144} = 10.62$, $P < 0.0001$; Minute Ventilation, $F_{2,24} = 36.69$, $P < 0.0001$, $F_{24,144} = 13.40$, $P < 0.0001$; Mean arterial blood pressure, $F_{2,24} = 8.96$, $P = 0.0012$, $F_{24,144} = 6.70$, $P < 0.0001$.

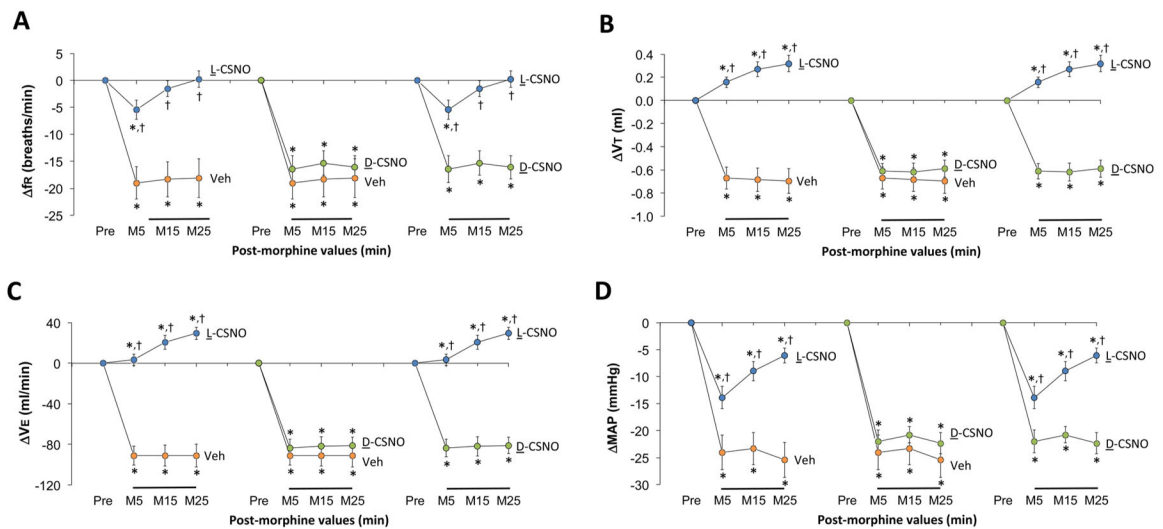


Fig. 2. Arithmetic changes in **(Panel A)** frequency of breathing (f_R), **(Panel B)** tidal volume (V_T), **(Panel C)** minute ventilation (V_E) and **(Panel D)** mean arterial blood pressure (MAP) elicited by bolus injection of morphine (2 mg/kg, IV) in rats undergoing continuous infusions of vehicle (VEH, 20 μ L/min, IV), S-nitroso-L-cysteine (L-CSNO, μ mol/kg/min, IV) or S-nitroso-D-cysteine (D-CSNO, 1 μ mol/kg/min, IV) in isoflurane-anesthetized rats. There were 9 rats in each group. Data are presented as mean \pm SEM. * $P < 0.05$, significant change from Pre. † $P < 0.05$, L-CSNO versus vehicle or D-CSNO. Repeated measures ANOVA statistics: Frequency of breathing, $F_{2,24} = 45.26$, $P < 0.0001$, $F_{24,144} = 5.66$, $P < 0.0001$; Tidal volume, $F_{2,24} = 43.30$, $P < 0.0001$, $F_{24,144} = 10.75$, $P < 0.0001$; Minute Ventilation, $F_{2,24} = 96.24$, $P < 0.0001$, $F_{24,144} = 5.55$, $P < 0.0001$; Mean arterial blood pressure, $F_{2,24} = 9.61$, $P = 0.0009$, $F_{24,144} = 7.24$, $P < 0.0001$.

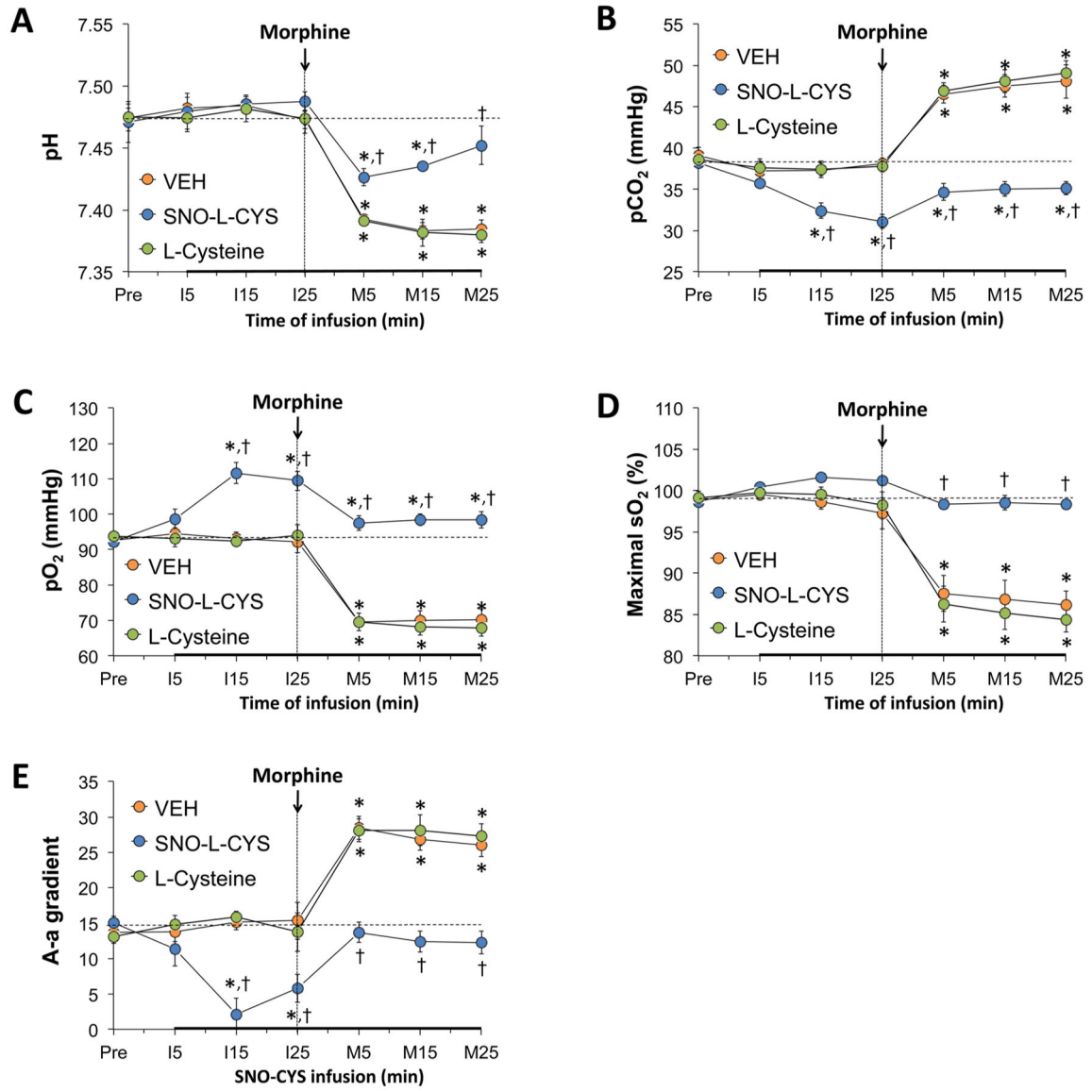


Fig. 3. (Panels A–D) Arterial blood-gas chemistry (pH, pCO₂, pO₂, maximal sO₂) and (Panel E) Alveolar-arterial (A-a) gradient values before (Pre), and during the continuous infusion (15 min onward) of vehicle (VEH, 20 μL/min, IV) or S-nitroso-L-cysteine (SNO-L-CYS, 1 μmol/kg/min, IV) or L-cysteine (1 μmol/kg/min, IV), and upon the bolus injection of morphine (2 mg/kg, IV; M5 onward) in isoflurane-anesthetized rats. There were 5 rats in each group. Data are presented as mean ± SEM. *P < 0.05, I15, I15 and I25 versus Pre and M5, M15, M25 versus I25. †P < 0.05, SNO-L-CYS or SNO-D-CYS versus vehicle. Repeated measures ANOVA statistics: pH, F_{2,24} = 3.86, P = 0.051, F_{12,84} = 4.95, P < 0.0001; pCO₂, F_{2,12} = 35.27, P < 0.0001, F_{12,72} = 5.07, P < 0.0001; pO₂, F_{2,12} = 43.13, P < 0.0001, F_{12,72} = 5.74, P < 0.0001; Maximal sO₂, F_{2,12} = 12.80, P = 0.0011, F_{12,72} = 8.82, P < 0.0001. A-a gradient, F_{2,12} = 20.96, P = 0.0001, F_{12,72} = 5.67, P < 0.0001.

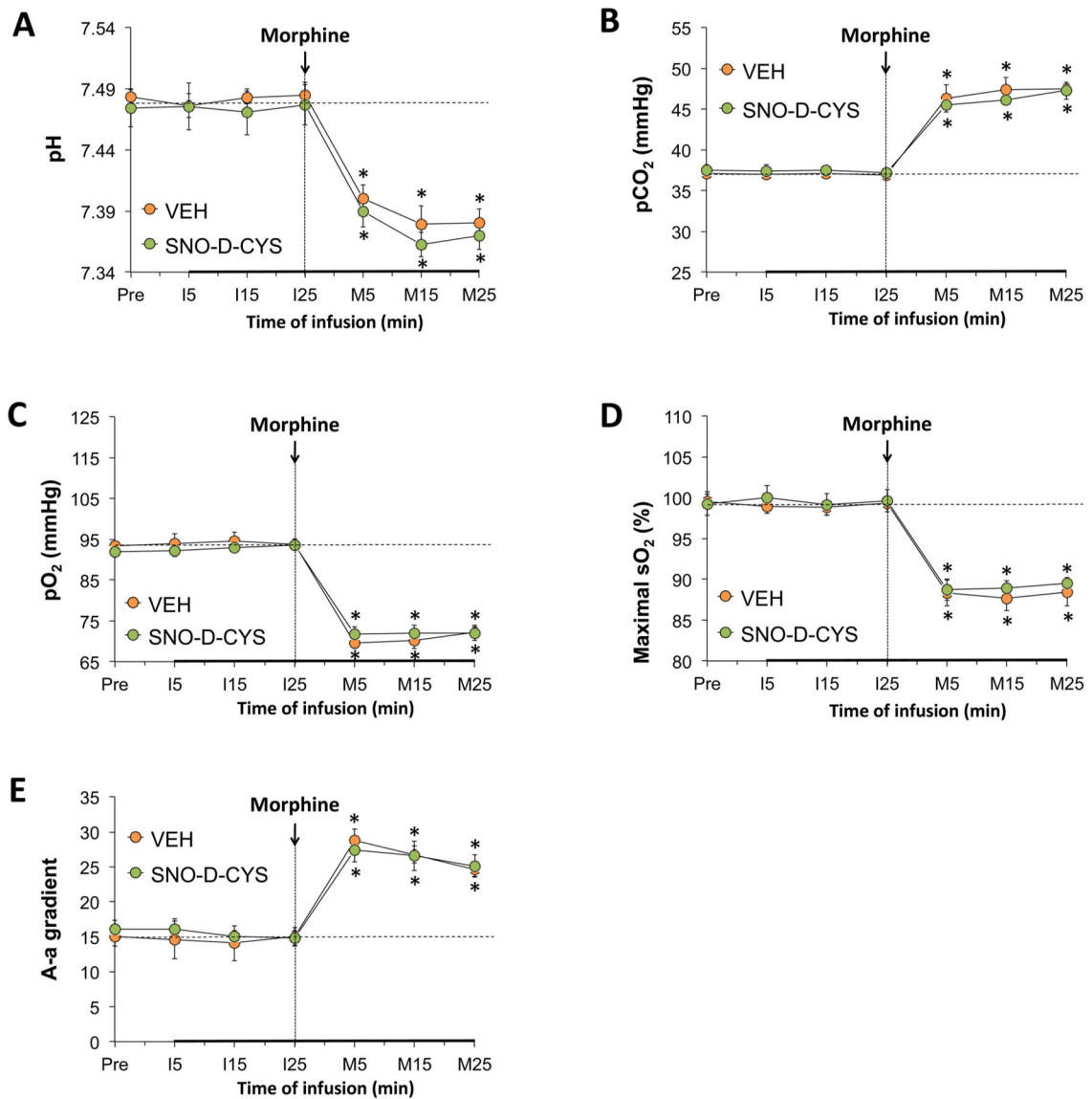


Fig. 4. (Panels A–D) Arterial blood-gas chemistry (pH, pCO₂, pO₂, maximal sO₂) and (Panel E) Alveolar-arterial (A-a) gradient values before (Pre), and during the continuous infusion (I5 min onward) of vehicle (VEH, 20 μL/min, IV) or S-nitroso-D-cysteine (SNO-D-CYS, 1 μmol/kg/min, IV), and upon the bolus injection of morphine (2 mg/kg, IV; M5 onward) in isoflurane-anesthetized rats. There were 6 rats in each group. Data are presented as mean ± SEM. *P < 0.05, I15, I15 and I25 versus Pre and M5, M15, M25 versus I25. †P < 0.05, SNO-L-CYS or SNO-D-CYS versus vehicle. Repeated measures ANOVA statistics: pH, F_{1,8} = 0.36, P = 0.56, F_{8,48} = 20.93, P < 0.0001; pCO₂, F_{1,8} = 0.02, P = 0.90, F_{8,48} = 6.69, P < 0.0001; pO₂, F_{1,8} = 0.01, P = 0.91, F_{8,48} = 10.08, P < 0.0001; Maximal sO₂, F_{2,12} = 0.04, P = 0.85, F_{8,48} = 6.92, P < 0.0001. A-a gradient, F_{1,8} = 0.29, P = 0.60, F_{8,48} = 4.33, P = 0.0006.

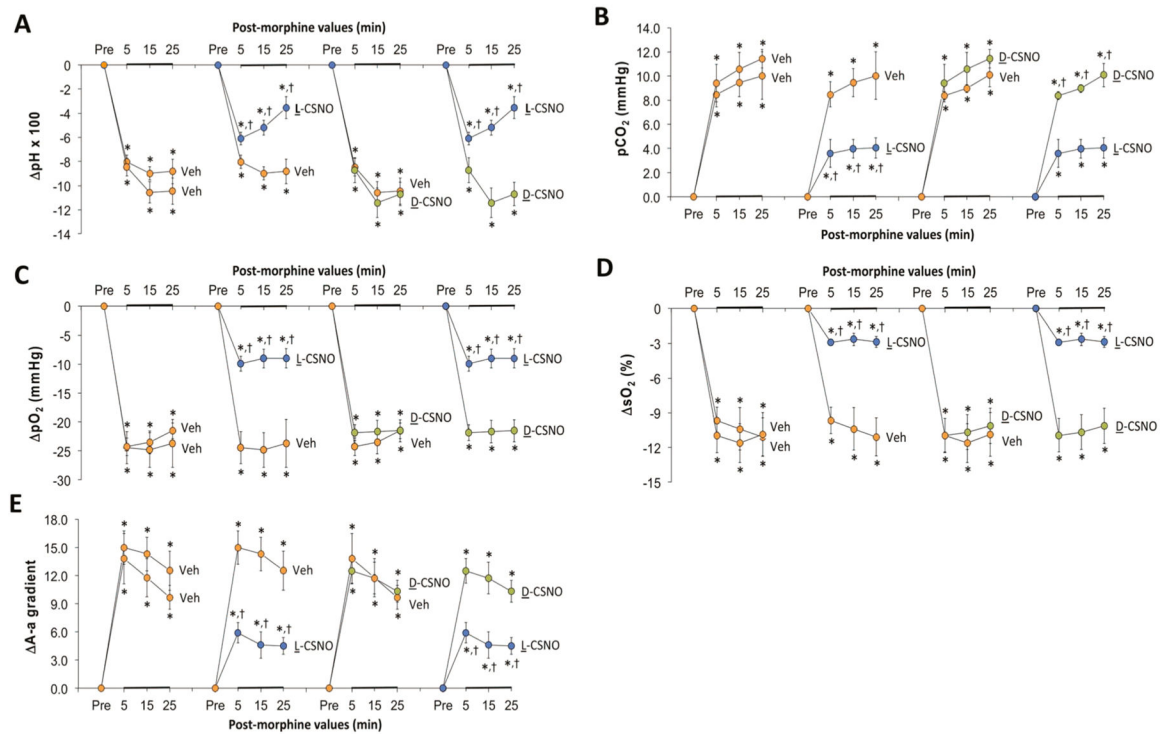


Fig. 5. Arithmetic changes in (**Panels A–D**) arterial blood-gas chemistry (pH, pCO₂, pO₂ and maximal sO₂) values and (**Panel E**) A-a gradient elicited by injection of morphine (2 mg/kg, IV) in rats receiving continuous infusions of vehicle (VEH, 20 μL/min, IV), S-nitroso-L-cysteine (L-CSNO, μmol/kg/min, IV) or S-nitroso-D-cysteine (D-CSNO, 1 μmol/kg/min, IV) in isoflurane-anesthetized rats. There were 6 rats in each group. Data are presented as mean ± SEM. *P < 0.05, significant change from Pre. †P < 0.05, L-CSNO versus vehicle or D-CSNO. Repeated measures ANOVA statistics: pH, F_{4,20} = 4.21, P = 0.01, F_{20,100} = 9.88, P < 0.0001; pCO₂, F_{4,20} = 18.59, P < 0.0001, F_{20,100} = 8.05, P < 0.0001; pO₂, F_{4,20} = 51.14, P < 0.0001, F_{20,100} = 4.33, P < 0.0001; Maximal sO₂, F_{4,20} = 10.22, P = 0.0001, F_{20,100} = 6.99, P < 0.0001; A-a gradient, F_{4,20} = 15.76, P < 0.0001, F_{20,100} = 4.84, P < 0.0001.

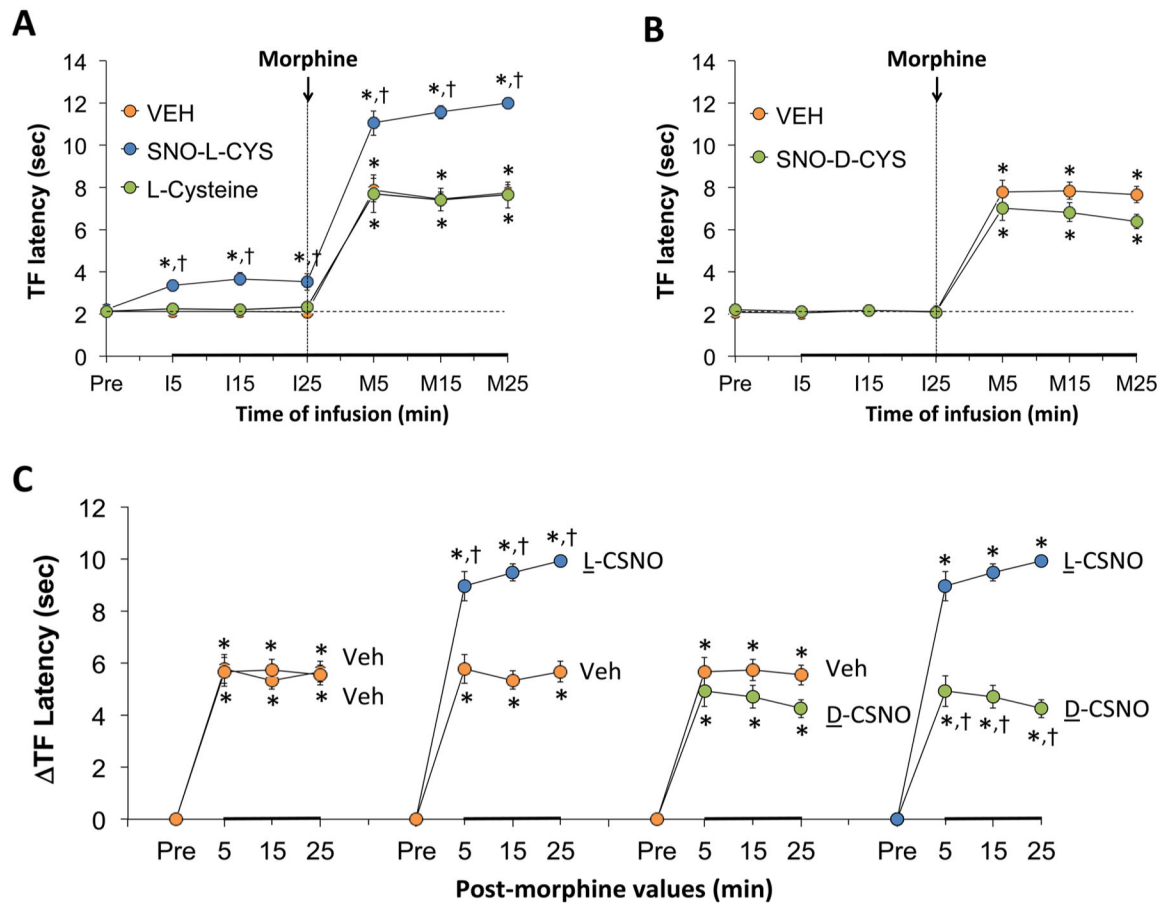


Fig. 6. Changes in tail-flick (TF) latency elicited by the continuous infusion (15 min onward) of **(Panel A)** vehicle (VEH, 20 μ L/min, IV) or S-nitroso-L-cysteine (SNO-L-CYS, 1 μ mol/kg/min, IV) or L-cysteine (1 μ mol/kg/min, IV) or **(Panel B)** vehicle (VEH, 20 μ L/min, IV) or S-nitroso-D-cysteine (SNO-D-CYS, 1 μ mol/kg/min, IV), and bolus injection of morphine (2 mg/kg, IV; M5 onward) in isoflurane-anesthetized rats. There were 9 rats in each group except for the L-cysteine group, which had 6 rats. Data are presented as mean \pm SEM. * $P < 0.05$, I15, I15 and I25 versus Pre and M5, M15, M25 versus I25. $\dagger P < 0.05$, SNO-L-CYS versus vehicle. **(Panel C)** Arithmetic changes in TF latency elicited by injection of morphine (2 mg/kg, IV) in rats undergoing continuous infusions of vehicle (VEH, 20 μ L/min, IV), S-nitroso-L-cysteine (L-CSNO, μ mol/kg/min, IV) or S-nitroso-D-cysteine (D-CSNO, 1 μ mol/kg/min, IV) in isoflurane-anesthetized rats. There were 9 rats in each group. Data are presented as mean \pm SEM. * $P < 0.05$, significant change from Pre. $\dagger P < 0.05$, L-CSNO versus vehicle or D-CSNO. Repeated measures ANOVA statistics for SNO-L-CYS study **(Panel A)**: $F_{2,21} = 64.60$, $P < 0.0001$, $F_{21,126} = 2.03$, $P = 0.0088$. Repeated measures ANOVA statistics for SNO-D-CYS study **(Panel B)**, $F_{1,16} = 2.70$, $P = 0.12$, $F_{16, 96} = 3.105$, $P = 0.0003$. Repeated measures ANOVA statistics for arithmetic changes in TF latencies (including the L-Cysteine group not shown in panels) **(Panel C)**: $F_{4,37} = 31.44$, $P < 0.0001$, $F_{37,185} = 3.27$, $P < 0.0001$.