Temporal increase in the reactivity of pulmonary vasculature to substance P in chronically hypoxic rats

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Lai, Yih-Loong, Szu-Jung Chu, Ming-Chieh Ma, and Chau-Fong Chen. Temporal increase in the reactivity of pulmonary vasculature to substance P in chronically hypoxic rats. Am J Physiol Regulatory Integrative Comp Physiol 282: R858–R864, 2002; 10.1152/ajpregu.00429.2001.—We previously demonstrated that the pulmonary vascular response to substance P (SP) increased in chronically hypoxic rats. This study explored the temporal increase in reactivity of the pulmonary vascular response to SP and its underlying mechanisms. First, young female Wistar rats were exposed to sea level (SL) or simulated high altitude (HA) for 15 h/day for 3 days, 1 wk, 2 wk, and 4 wk. Lungs were isolated and perfused with 4% bovine serum albumin in Krebs-Henseleit buffer solution. SP (1.5 × 10^-4 M) induced significant increases in pulmonary arterial pressure (Ppa), venous pressure (Pv), capillary pressure (Pc), arterial resistance (Rpa), and filtration coefficient (Kfc) in SL lungs. Increases in Ppa and Rpa were significantly augmented in HA lungs, with a temporal increase trend peaking at 2 wk of HA exposure. The selective neurokinin (NK) type 1 (NK1) receptor antagonist SR-14033 significantly attenuated SP-induced increases in Ppa, Pc, Rpa, Rpa, and Kfc in SL lungs. In lungs exposed to HA for 2 wk, SR-14033 suppressed the effect of SP on Ppa. Also, chronic hypoxia induced significant increases in NK1 receptors and NK1 receptor mRNA, with a temporal trend. We conclude that chronic hypoxia temporally augments SP-induced vascular responses, which are closely associated with increases in NK1 receptors and gene expression.

MATERIALS AND METHODS

This study was performed in three parts. Part 1 was carried out to explore temporal changes in pulmonary vascular reactivity to SP in chronically hypoxic rats. Part 2 was performed using the antagonist of SP. In part 3, we analyzed the NK1 receptor and NK1 receptor gene expression.

Animal preparation. In part 1, 29 young female Wistar rats weighing 219 ± 3 g were divided into five groups: sea level (SL; n = 6), hypoxia for 3 days (3D-HA; n = 5), hypoxia for 1 wk (1W-HA; n = 5), hypoxia for 2 wk (2W-HA; n = 6), and hypoxia for 4 wk (4W-HA; n = 6). Animals in the SL and 2W-HA groups were treated as described above. Each group was further evenly divided into three subgroups: SP, SR-14033 + SP, and SR-48968 + SP.

For part 2, 36 young female Wistar rats weighing 234 ± 3 g were evenly divided into two groups: SL (n = 18) and 2W-HA (n = 18). The SL and 2W-HA animals were treated as described above. Each group was further evenly divided into three subgroups: SP, SR-14033 + SP, and SR-48968 + SP.

For part 3, we analyzed the NK1 receptor and NK1 receptor gene expression in 20 young animals evenly divided into four groups: SL, 1W-HA, 2W-HA, and 4W-HA. For exposure to air (SL) or hypoxia, the animals were treated as described for part 1.

Setup for the isolated perfused lungs. After a fixed period of air or hypoxic exposure, each animal was anesthetized with pentobarbital sodium (40 mg/kg ip). Then the isolated perfused lungs were prepared as described by Huang and Lin (10), with some modifications. Briefly, after insertion of a tracheal cannula, the chest was opened and the lungs were ventilated with humidified 95% air-5% CO2 under an end-expiratory pressure of 2.5 cmH2O. After the right ventricle was injected with heparin (150 IU), the pulmonary artery was cannulated and perfused with a perfusate. The perfusate was a mixture of bovine serum albumin (4 mg/100 ml) and Krebs-Henseleit buffer solution containing (in mM) 118 NaCl, 4.7 KCl, 2.5 CaCl2-H2O, 1.2 MgSO4-7 H2O, 1.2 KH2PO4, 25 NaHCO3, and 10 glucose. A wide-bore cannula

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was placed in the left atrium through the left ventricle to collect the effluent perfusate for recirculation. About 50 ml of initial perfusate were discarded to clear the blood before initiation of recirculation. A perfusion rate of 3 ml·min⁻¹·100 g body wt⁻¹ was maintained by a roller pump through an air bubble trap. The heart and lungs were removed en bloc and placed on a weighing pan, which was mounted on a Grass force transducer for detecting the change in lung weight and was suspended in a constant-temperature (37°C) humidified chamber. The weighing system was calibrated by placing a 2-g weight on the pan and adjusting the output to 5 cm of chart deflection. The pulmonary arterial (Pp) and venous pressures (Pv) were continuously monitored with Statham pressure transducers, which were placed at the same height as the heart. Distances between the pressure transducers and the pulmonary artery and vein were 29 and 50 cm, respectively. Resistances of the connecting catheters were measured, and then the above-measured Pp and Pv were corrected for these resistances of connecting tubings. Changes in lung weight, Pp, and Pv were continuously recorded with a Grass recorder. In addition, the isolated perfused lungs were continuously ventilated with 95% air-5% CO₂.

Capillary pressure. With a constant-flow perfusion, venous outflow was momentarily stopped for 3–4 s at end expiration. There was a rapid rise in Pp, followed by a slower but steady rise. Capillary pressure (Pc) was obtained by extrapolating the slow-rising component back to time 0 (3).

Filtration coefficient. The filtration coefficient (Kf) was determined by the gravimetric method of Drake et al. (4). On achieving an isogravimetric state, we raised the Pc, rapidly by 10 cmH₂O for 10 min. This hydrostatic pressure caused the lung to gain weight promptly. This was followed by a slow but steady rise in lung weight. The rapid component represents the expansion of pulmonary blood vessels, whereas the slow component is due to fluid filtration into the interstitial space. The initial rate of fluid filtration was estimated by extrapolating the slow component to time 0 in a semilogarithmic plot. The value of the y-intercept was divided by the hydrostatic pressure change (ΔPc) and normalized to 100 g of lung weight.

Experimental protocols. In part 1, we studied the pulmonary vascular response to SP. In a preliminary study, the dose-dependent increase of Pp in response to SP was analyzed. At the end of a 20-min equilibration (baseline) period, baseline values of vascular parameters were measured. Then SP (5 × 10⁻⁵, 1.5 × 10⁻⁴, or 2.5 × 10⁻⁴ M) was added to the perfusate, and the vascular response to SP was recorded. At 10 min after the first addition of SP or when pulmonary vascular parameters returned to baseline, the perfusate was changed and a new SP solution was added to the perfusate. Subsequently, with the same procedure, SP-induced responses were tested again using another SP solution. According to this preliminary study, 1.5 × 10⁻⁴ M SP (unless otherwise noted) was chosen for vascular challenge in the subsequent experiments.

In the isolated perfused SL or HA rat lung, the general protocol for SP challenge was a 20-min equilibration (baseline) period and an SP (1.5 × 10⁻⁴ M) challenge period. Pp, Pv, Pc, and change in lung weight were determined before (baseline period) and after the SP challenge. Then arterial resistance (Rpa), venous resistance (Rv), and Kf were calculated separately.

For part 2, the general protocol for SP challenge was a 20-min equilibration (baseline) period, a 10-min antagonist (10⁻7 M SR-14033 or 10⁻⁷ M SR-68968) period, and the SP (10⁻⁴ M) challenge period. Pp, Pv, Pc, and Kf were determined before (baseline period) and after the SP challenge. Then Rpa, Rv, and Kf were calculated separately.
The total NK₁ receptor RNA preparation from the lung tissues of the SL and HA rats was extracted using TRIzol reagent (GIBCO BRL, Grand Island, NY) following the manufacturer’s instructions. The exact amount of total extracted RNA was determined by optical density for each sample, and its quality was confirmed by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. Its quality was confirmed by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. RNA was determined by optical density for each sample, and the manufacturer’s reagent (GIBCO BRL, Grand Island, NY) following the manufacturer’s instructions.

The PCR procedure for amplification reactions was performed in a 50-μl final volume containing 5× TaqMan buffer, 3.0 mM Mn(OAc)₂, dNTP (0.3 mM each), 0.5 U of AmpErerase uracil N-glycosylase (1 U/μl), and 5.0 U of rTth DNA polymerase (2.5 U/μl) from the TaqMan EZ RT-PCR kit (Perkin-Elmer). The final concentration of NK₁ receptor and GAPDH gene forward and reverse primers was 4.1 μM. The final concentration of NK₁ receptor and GAPDH gene fluorogenic probes was 2.05 μM. To reduce variability between replicates, PCR premixes, which contained all reagents except total RNA, were prepared and aliquoted into 1.5-ml microtubes. Forty microliters of the reaction mixture were added to the PCR optical tube (MicroAmp, Perkin-Elmer) containing 10 μl of 200 ng of unknown sample RNA or ileac RNA, termed the calibrator sample. Thermal cycling conditions were 2 min at 50°C and 30 min at 60°C followed by 5 min at 95°C and 50 cycles of 15 s at 94°C and 1 min at 60°C.

**Statistical analysis.** Values are means ± SE. Differences in parameters among groups were analyzed with analysis of variance. If significant differences existed among groups, statistical differences between any two groups were analyzed by the Newman-Keuls test. Differences were considered significant if P < 0.05. Differences between values before and after the SP challenge were analyzed by paired t-test.

The comparative Ct (ΔΔct) method was used to quantify NK₁ receptor mRNA levels (13, 24). The advantage of this method is that it eliminates the need for standard curves. The ΔΔct method uses a single sample, termed the calibrator sample, for comparison of every unknown sample’s gene expression level. The calibrator sample is analyzed on every assay plate with unknown samples of interest. We used the mRNA expression of the rat ileum as the calibrator sample to represent onefold expression of the gene of interest. The calculation is as follows: ΔΔct = [Ct NK₁ (unknown sample) – Ct GAPDH (unknown sample)] – [Ct NK₁ (calibrator sample) – Ct GAPDH (calibrator sample)]. The formula that can be used is as follows: fold induction = 2^{-ΔΔct} (24).

**RESULTS**

**Part 1.** Compared with the baseline value, two doses of SP (1.5 × 10^{-4} and 2.5 × 10^{-4} M) caused a significant increase in Ppa (Fig. 1). Because 1.5 × 10^{-4} M SP caused a prominent increase in Ppa, we used this dose for the subsequent SP challenges.

Challenge with 1.5 × 10^{-4} M SP caused a marked increase in Ppa in all groups. Compared with SL lungs, the SP challenge induced significant increases in Ppa (Fig. 2) and Rₐ (Fig. 3) in all hypoxic lungs. In addition, hypoxia-augmented, SP-induced increases in Ppa and Rₐ showed a temporal increasing trend, with a peak response at 2 wk of hypoxia (2W-HA). Also, a hypoxia-augmented, SP-induced increase in Ppa, was found in 3D-HA and 1W-HA lungs (Fig. 2). On the other hand, chronic hypoxia did not significantly augment the SP-induced increase in Ppa (Fig. 2).

The SP challenge caused a marked increase in Kₑ in SL lungs. This SP-induced increase in Kₑ was significantly reduced in all HA lungs (Fig. 4).

**Part 2.** In SL lungs, SR-14033 significantly attenuated SP-induced increases in Ppa (Fig. 5), Pₑ (Fig. 5), and Kₑ (Fig. 6). However, SR-48968 did not induce any significant effect on SP-induced alterations in pulmonary vascular parameters, except Pₑ. For HA lungs, SR-14033 significantly attenuated the SP-induced increase in Ppa (Fig. 7). On the other hand, SR-48968 did not cause any significant alteration in SP-induced increases in vascular parameters.

**Part 3.** The NK₁ receptor antiserum recognized broad protein bands of 70 to >120 kDa in membranes prepared from the rat ileum (Fig. 8A, lane 1). The pattern of the protein bands in membranes prepared from lung tissue in the SL rats was the same as that in membranes prepared from the ileum (Fig. 8A, lane 2). These protein bands were detected strongly in the membranes prepared from HA rats (Fig. 8A, lanes 3–5). Figure 8B shows the semiquantitative density of the major 79-kDa band in both groups. The integrated digital values per counting area were significantly higher in 1W-HA, 2W-HA, and 4W-HA groups (107.7 ± 8.5, 121.3 ± 8.5, and 123.2 ± 5.8, respectively) than in the SL group (91.7 ± 7.1; Fig. 8). Hypoxia induced a significant increase in NK₁ receptor mRNA (Fig. 9); the increase reached a maximal value in the 4W-HA group.

**DISCUSSION**

We demonstrated SP-induced increases in Ppa, Pₑ, Pᵥ, Kₑ, and Rₑ in isolated perfused SL lungs. Prolonged exposure to hypoxia augmented the SP-induced increases in Ppa, Pₑ, and Rₑ, with a temporal increase trend peaking at 2 wk. On the other hand, chronic
hypoxia significantly attenuated the SP-induced increase in $K_{fc}$. SP-induced increases in vascular responses were significantly attenuated by SR-14033 in SL lungs. Also, SR-14033 significantly attenuated the SP-induced increase in $P_{pa}$ of HA lungs. In addition, chronic hypoxia induced significant increases in NK$_1$ receptors and NK1 receptor mRNA. Several features of the relationship between prolonged hypoxia and SP-induced pulmonary vascular alterations are discussed below.

**Vascular alterations caused by SP in isolated perfused rat lungs.** SP increased $P_{pa}$, $P_v$, $P_c$, $R_a$, and $K_{fc}$ in the isolated perfused SL lungs. These pulmonary vascular alterations were similar to those observed in the isolated perfused guinea pig lung (20) and the isolated rabbit pulmonary arterial strip (23). However, the dose of SP needed to produce a marked increase in $P_{pa}$ was much larger than that required in the isolated guinea pig lung (20) or the isolated rabbit pulmonary arterial strip (23). This difference might be due mainly to fewer NK$_1$ receptors in the rat lung than in the guinea pig lung (8). In addition, there might be a difference in NK$_1$ receptors between the rat and the guinea pig lungs (8).

A much higher dose of SP was also required to cause vascular constriction in the isolated perfused lungs than in the in vivo preparation (2, 22). This may be due to a difference caused by different preparations. The doses of agonists required to induce pharmacological responses in the in vitro isolated perfused lung are often larger than those in the in vivo preparation (12). Because the in vitro lung was perfused with the phys-
iological salt solution without blood, the higher vascular reactivity to SP in vivo might relate, partly at least, to a component(s) of blood.

In SL and HA lungs, SP induced a large increase in $P_{pa}$ and a small elevation in $P_c$ (Fig. 2). Therefore, it is tempting to reason that SP causes mainly a constriction in the arterial segment of the pulmonary vasculature.

**Temporal increases in SP-induced vascular reactivity of HA rats.** After prolonged exposure to hypoxia, SP-induced increases in $P_{pa}$ and $R_a$ were augmented (Fig. 2). In addition, this augmentation increased gradually with exposure time until 2 wk of hypoxia. No significantly temporal augmentation of SP-induced increase in $P_v$, $P_c$, or $K_{fc}$ was found, however. We reasoned that several factors may cause this temporal augmentation during chronic hypoxia. 1) There is a temporal increase in NK1 receptors during the process of hypoxia (Fig. 8). 2) Prolonged hypoxia may induce a change in neutral endopeptidase (NEP; the major degradation enzyme of SP) activity. It is known that oxygen radical production increases during hypoxic exposure (16) and that NEP activity is inhibited by oxygen radicals. Therefore, the same dose of SP could become more potent because of a decrease in NEP activity followed by prolonged hypoxia. 3) SP may induce releases of more constrictors in the lungs after chronic hypoxia. Previous studies have shown that SP may release thromboxane (20) and leukotrienes (5). These releasing mechanisms may be enhanced, since oxygen radical production increases during prolonged hypoxia. 4) There is a controversy over whether endothelium-derived nitric oxide activity is increased or decreased in the hypertensive pulmonary vasculature of chronically hypoxic rats (1, 19, 21, 25). It is possible that prolonged hypoxia induces an impaired endothelium-dependent relaxant activity (1) and a decrease in nitric oxide production in the pulmonary vasculature (21). Then the impaired endothelial function should augment SP-induced vasoconstriction. 5) The wall thickness of pulmonary vessel increased after prolonged hypoxia (18). The same dose of SP should induce a larger response in the vessel with more smooth muscle mass and, thus, an increase in SP reactivity.

Contrary to the changes in vascular pressures, SP caused a large increase in $K_{fc}$ in SL lungs but only a small increase in HA lungs (Fig. 4). In other words, the SP-induced marked elevation in $K_{fc}$ in SL lungs was significantly reduced in all HA lungs. Accordingly, these data may imply that there should be a marked decrease in vascular leakage after prolonged exposure to hypoxia. This is compatible with the fact that HA pulmonary edema during reascent is rare when the SL sojourn is short (<10–14 days) (11).

NK1 receptors in the augmented vascular response to SP in HA rats. Because the action of SP is mediated mainly via NK1 and much less via NK2 receptors (15), we employed the NK1 receptor antagonist SR-14033 and the NK2 receptor antagonist SR-48968 to test their blocking action on SP-induced vascular reactivity. In SL lungs, SR-14033 significantly attenuated SP-induced increases in $P_{pa}$, $P_v$, $P_c$, $R_a$, and $K_{fc}$ (Figs. 5 and 6).

![Fig. 5. SP-induced percent changes in $P_{pa}$, $P_v$, and $P_c$ in rats exposed to SL in the presence or absence of SR-14033 or SR-48968. *Significantly different ($P < 0.05$) from SP alone.](http://ajpregu.physiology.org/)

![Fig. 6. SP-induced percent change in $K_{fc}$ in the presence or absence of SR-14033 or SR-48968 in rats exposed to SL and 2 wk of hypoxia. *Significantly different ($P < 0.05$) from SP alone.](http://ajpregu.physiology.org/)

![Fig. 7. SP-induced percent changes in $P_{pa}$, $P_v$, and $P_c$ in the presence or absence of SR-14033 or SR-48968 in rats after 2 wk of hypoxia. *Significantly different ($P < 0.05$) from SP alone.](http://ajpregu.physiology.org/)
6), while SR-48968 significantly attenuated the increase in $K_{fc}$ (Fig. 6). In HA lungs, SR-14033 significantly attenuated the SP-induced increase in $P_{pa}$ (Fig. 7), while SR-48968 did not significantly attenuate any SP-induced response. Thus it is clear that the action of SP is mediated mainly via the NK1 receptor in SL lungs, while the same action is mediated almost exclusively via the NK1 receptor in HA lungs. Fewer SP-induced vascular alterations were significantly attenuated by SR-14033 in HA than in SL lungs. This difference between SL and HA lungs may be explained by the fact that HA lungs have more NK1 receptors and, thus, are less suppressed by SR-14033 than SL lungs.

Results from Western blotting (NK1 receptors) and real-time RT-PCR NK1 receptor gene expression experiments suggest that the increased density of NK1 receptors after prolonged hypoxia may be due to increased transcription of the NK1 receptor gene. Therefore, our data support the conclusion that the increase in the pulmonary vascular response to SP induced by chronic hypoxia can be attributed to an upregulation of NK1 receptors during the hypoxic exposure process. It is not clear why there is an upregulation of NK1 receptors in the lungs of chronically hypoxic rats. We speculate that this upregulation might relate to hypoxia-inducible factor, reactive oxygen species, leukotrienes, and endothelins. Further studies are needed to delineate the speculated effects of these possible factors.

**Perspectives**

In 1995, using capsaicin to deplete tachykinins, we found that tachykinins play an important role in chronic hypoxic pulmonary hypertension (14). However, capsaicin depletes several neuropeptides in afferent C-fibers, including bombesin, calcitonin gene-related peptide, somatostatin, and tachykinins (9). Compared with other neuropeptides in the afferent C-fibers, tachykinins may induce different responses in the pulmonary circulation. To carry out more specific studies related to the role of tachykinins in chronic hypoxic pulmonary hypertension, we started to use specific tachykinin receptor agonists and antagonists to demonstrate that tachykinin NK1 and/or NK2 receptors are involved in chronic hypoxic pulmonary hypertension in vivo (2). This study is a continuation of this series of studies. Using in vitro experiments, we demonstrated that the temporal increase in pulmonary vascular reactivity to SP was closely related to an increase in NK1 receptor gene expression during exposure to chronic hypoxia.

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