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Original Article

The Interaction between Trolox and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic Acid on Hypoxic Pulmonary Vasoconstriction in the Isolated Rabbit Lung

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Abstract

Background: The mechanism of hypoxic pulmonary vasoconstriction (HPV) is still debatable. It has been proposed that reactive oxygen species (ROS) might be involved in HPV. However, there is no special transporter for superoxide anion in the cell membrane and it may release from the cells via anion exchanger. Therefore, the aim of this study was to investigate the interaction of ROS and anion exchanger in acute HPV.

Methods: The present study was performed in the isolated rabbit lung. After preparation, the lungs were divided into four hypoxic groups of control, Trolox (antioxidant)-treated, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, anion exchanger inhibitor)-treated, and Trolox+DIDS-treated. Pulmonary artery pressure, left atrial pressure, and lung weight were continuously registered and PVR was then calculated. PO_2, PCO_2, HCO_3^-, pH, and NO metabolites of the perfusate were measured during steady-state and at the end of experiments (30 minutes). All data were compared with ANOVA and t-test and significance was considered when $P<0.05$.

Results: Ventilation of the lungs with hypoxic gas induced HPV in the control group. DIDS did not have a further effect on HPV compared with the control group. The combination of Trolox and DIDS decreased HPV rather than Trolox per se at 5 minutes. Furthermore, HPV was abolished in both the Trolox and Trolox+DIDS groups at 30 minutes. Concentrations of NO metabolites in the Trolox+DIDS group were more than other groups.

Conclusion: The present study indicates a possible interaction between ROS and anion exchanger in acute HPV. It also suggests the modulatory effect of NO at above condition.

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What's Known

- Although constriction of pulmonary vessels in response to alveolar hypoxia (HPV) has been demonstrated previously, but its mechanism remains debatable.

What's New

- There is a possible interaction between reactive oxygen species and anion exchanger in HPV by a mechanism partly linked to NO production.

Introduction

Some respiratory diseases may lead to local or global alveolar hypoxia. However, the exact mechanisms of vasoconstriction of pulmonary vessels during hypoxia (HPV) have not been identified yet.[1,2]

Many investigators have reported the role of reactive oxygen species (ROS) in HPV. However, there are conflicting results on whether ROS decreases or increases during HPV.[1,3-6] Furthermore, the contribution of ROS in the acute and sustained phases of HPV is still arguable.[7] Superoxide anion (O_2^-) is one of the oxygen free radicals which is supposedly involved in HPV.[1] O_2^- does not easily pass through the pulmonary smooth muscle cell membrane. Therefore, it should be eliminated from the cells by a carrier or an anion channel. So far, no special carrier is known for O_2^-. However, the rate of O_2^- release from the isolated pulmonary rat artery increases during short-term hypoxia in the presence of bicarbonate buffer.[8] Furthermore, inhibition of Cl-HCO_3^- exchanger (anion exchanger, AE) prevents the release of O_2^- in rat's pulmonary artery and isolated rabbit lung.[8,9] These data indicate that O_2^- may exit from the cells by AE. Two isoforms of AE, Na-dependent, and Na-independent were detected in the pulmonary vessels. Na-dependent AE leads HCO_3^- anions to enter the cells in exchange for chloride anions. The activity of this transporter increases during hypoxia and leads to intracellular alkalinization in the pulmonary smooth muscle cells.[10,11] Therefore, HCO_3^- may enter into the smooth muscle cells in exchange for O_2^- instead of chloride anion during hypoxic condition.[8]

Some studies have reported the role of extracellular HCO_3^- on the regulation of pulmonary vascular tone. Hypercapnia with normal pH (corrected by bicarbonate) is indicated to increase pulmonary artery wall tension in rats.[12] Also, pulmonary artery pressure increases during hypoxia and hypercapnia with pH adjusted by HCO_3^- in the isolated perfused rabbit lung.[13,14] A few studies have addressed the role of AE on the regulation of pulmonary vascular resistance. DIDS, acetazolamide (carbonic anhydrase inhibitor), and sodium-free buffer decrease pulmonary vascular tone during hypercapnia with normal pH, which suggests the role of Na-dependent AE on the pulmonary vasculature.[12] Moreover, high dose of DIDS prolongs acute phase and prevents sustained phase of HPV in the isolated rabbit lung, which proposes the different role for AE in acute and sustained phases of HPV.[15] However, it is not fully clear by which mechanism AE may regulate pulmonary vascular tone, and whether changes in intracellular pH or entrance of HCO_3^- have an important role in HPV. Furthermore, the interaction of HCO_3^- and O_2^- at above condition is unknown.

There is a general agreement that nitric oxide (NO) is involved in the regulation of pulmonary vascular tone. Both in vivo and ex vivo studies have revealed that NO production decreased in HPV.[16,17] Therefore, at least a part of vasoconstriction during hypoxic gas ventilation is in concomitant with the reduction of NO production, which could influence superoxide release too.[18,19] Many studies indicated interactions between O_2^- and NO in the lung during normoxic and hypoxic conditions. Activation of Na-dependent AE may decrease NO availability by increasing O_2^- release. Inhibition of each may increase the availability of the other and induce pulmonary vasoconstriction by O_2^- or pulmonary vasodilatation by NO.[20,21] Therefore, it may be concluded that DIDS increases NO production by preventing O_2^- release through the smooth muscle cells and thereby decreasing HPV. However, no study has indicated the interaction between ROS and anion exchanger and a possible role of NO during ventilation of the lungs with hypoxic gas.

With the above background, this study was performed to clarify the interaction of O_2^- and AE in HPV. Therefore, we added DIDS as an AE inhibitor and antioxidant Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, to the perfusate before hypoxic gas ventilation in the isolated rabbit lung and measured changes in pulmonary artery pressure during the time course of experiments. Furthermore, in order to reveal the effects of Trolox or/and DIDS on NO production, we measured NO metabolites in the perfusate at above conditions.

Materials and Methods

Lung Isolation, Perfusion, and Ventilation

Experiments were approved by the Center of Comparative and Experimental Medicine and the Ethical Committee of Animal Care at Shiraz University of Medical Sciences, Shiraz, Iran. Animals were housed in standard cages under controlled laboratory conditions of temperature, humidity, and 12:12-hour light-dark cycle. They had free access to water and standard food a few days before starting the experiments. The model of isolated rabbit lungs has been described previously.[15,22] Briefly, 20 male New Zealand rabbits with body weight (BW) of 2.03±0.07 kg were anaesthetized by i.v. administration of ketamine (30-50 mg/kg BW) and xylazine (6-10 mg/kg BW) and heparinized (1500 U/kg BW).[13,14] The trachea was cannulated and animals were ventilated with room air (tidal volume: 18.48±0.58 ml, respiratory rate: 30/min). Chest wall was opened and about 40 ml heparinized blood was collected by cardiac puncture. The blood was then centrifuged at 4 °C at 4000 g for

7 minutes and about 20 ml of plasma was stored in ice. Then, pulmonary artery was cannulated and the lung perfused with 4 °C air bubble-free Krebs-Henseleit solution (perfusate) through the pulmonary artery cannula connected to a peristaltic pump with a flow rate of 20 ml/min. Next, the left atrium was cannulated. Finally, isolated ventilated perfused lung was placed in a temperature-equilibrated housing chamber and freely suspended from a force transducer and recirculation was performed. The whole system was heated from 4 °C to 38 °C. Meanwhile, the flow rate was slowly increased from 20 to 140 ml/min. The left atrial pressure and positive end-expiratory pressure (PEEP) were set at 1.5-2.5 cm H_2O and 2 cm H_2O, respectively. After 20 minutes of steady state period, the perfusate was changed, plasma was added to the perfusate, and the lung was stabilized for additional 20 minutes. All lungs included in the study exhibited criteria similar to our previous studies.[13,14,23]

Compositions of Gases and Perfusate

Two different gas mixtures were employed in this study; (i) normoxic normocapnic gas: 21% O_2, 5.5% CO_2 balanced with N_2 and (ii) hypoxic normocapnic gas: 3% O_2, 5.5% CO_2 balanced with N_2. The perfusate used in this study contained in mM: 120 NaCl, 1.1 K_2HPO_4, 1.3 Mg PO_4, 4.3 KCl, 2.4 $CaCl_2$, and 13.32 glucose. pH was adjusted to the normal range of 7.35-7.40 with $NaHCO_3$ in all experimental groups.

Study Protocol

After 40 minutes of the steady-state period, lungs were ventilated in a humidified chamber with hypoxic gas for 10 minutes to evaluate lung viability and its responses to short alveolar hypoxia as a routine test procedure. Thereafter, the lungs were ventilated with normoxic gas for 20 minutes. Subsequently, the lungs were randomly divided into four groups, namely control (HOX, n=5), DIDS (200 µM, Sigma, n=5) treated,[9] Trolox (20 µM, Sigma, n=5) treated,[24] and Trolox+DIDS treated group (n=5). Trolox and/or DIDS were solved and added to the perfusate 10 minutes before the onset of the hypoxic gas ventilation. Pulmonary artery pressure, left atrial pressure, airway pressure, and lung weight were continuously registered using a data acquisition system (Power lab, AD instrument, Australia) connected to a pressure and force transducers. PVR was calculated from pulmonary artery pressure, left atrial pressure, and perfusate flow rate by using Ohm equation. ΔPVR was also calculated to evaluate changes in PVR irrespective of its basal values in the experimental groups. Perfusate samples were taken during steady-state and at 30 minutes in order to measure PO_2, PCO_2, HCO_3^-, pH, and NO metabolites of each group.

Analysis of NO Metabolites

Griess Method was used to measure NO metabolites of the perfusate as described earlier.[25] Standard solutions were prepared with different concentrations of sodium nitrite. Perfusate samples were taken from pulmonary venous outflow and were stored at -80°C until measurement. After incubating the samples and standards by Griess reagent and vanadium chloride in a 96-well plate, the absorbance of samples was detected at 540 nm wavelengths using a microplate reader (Biotek, USA) and the concentrations of NO metabolites were calculated.

Statistical Analysis

Data are presented as mean±SE. The analysis of variance (ANOVA) with LSD post hoc test was used for the comparison of means between the experimental groups. Paired t-test was used for comparison of the values in each group at 5 and 30 minutes. All analysis was performed using the SPSS software, version 18.0. $P<0.05$ were considered statistically significant.

Results

There was no significant difference between PO_2, PCO_2^-, and pH in all hypoxic groups at 30 minutes of the experiments. However, concentration of HCO_3^- in the Trolox group was lower compared to the control (P=0.006) and DIDS (P=0.05) groups. Also, HCO_3^- in the Trolox+DIDS group was less than the control (P=0.001) and DIDS (P=0.011) groups (table 1).

There was no difference between ΔPVR in the control and DIDS groups at 5 minutes of the experiments (P=0.667). The administration of Trolox decreased PVR compared to the control (P=0.016) and DIDS groups (P=0.007). PVR in the Trolox+DIDS group was also lower than the control (P=0.001) and DIDS (P<0.001) groups. Furthermore, PVR in the Trolox+DIDS group was insignificantly less as compared with the Trolox group (P=0.130) (figure 1).

There was no difference between ΔPVR in the control and DIDS groups at 30 minutes of the experiments (P=0.457). However, PVR decreased in the Trolox group compared to the control (P=0.005) and DIDS (P=0.020) groups. Similar reduction in PVR occurred in the Trolox+DIDS group compared to the control (P=0.005) and DIDS (P=0.022) groups. There

Table 1: Gas parameters in the perfusate at 30 minutes of hypoxic gas ventilation in the experimental groups

	Control	DIDS	Trolox	Trolox+DIDS	P value between groups
pH	7.39±0.01	7.37±0.01	7.36±0.02	7.37±0.03	0.677
PCO_2 (mmHg)	33.21±1.26	37.15±0.33	35.66±2.05	34.83±2.07	0.717
PO_2 (mmHg)	34.71±1.05	34.50±0.60	34.40±1.14	35.00±2.33	0.756
HCO_3- (mmol/L)	21.20±0.18	21.58±0.12	20.06±0.30**##	20.33±0.24*#	0.004

Data are presented as mean±SE (n=5 in each group); **P<0.01 in Trolox vs. control group; ##P<0.01 in Trolox vs. DIDS group; *P<0.05 in Trolox+DIDS vs. control group; #P<0.05 in Trolox+DIDS vs. DIDS group

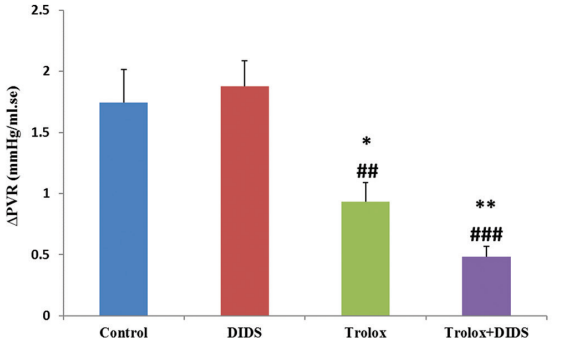

Figure 1: Pulmonary vascular resistance (ΔPVR) in Trolox-treated and Trolox+DIDS-treated groups were lower than other groups at 5 minutes. Data are presented as mean±SE (n=5 in each group). *P<0.01 in Trolox vs. control group; **P<0.01 in Trolox+DIDS vs. control group; ##P<0.01 in Trolox vs. DIDS group; ###P<0.001 in Trolox+DIDS vs. DIDS group.

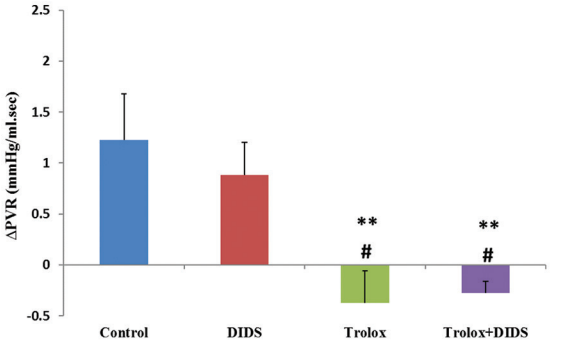

Figure 2: Pulmonary vascular resistance (ΔPVR) in Trolox-treated and Trolox+DIDS-treated groups were lower than other groups at 30 minutes. Data are presented as mean±SE (n=5 in each group). **P<0.01 at each group vs. the control group; #P<0.05 at each group vs. the DIDS group.

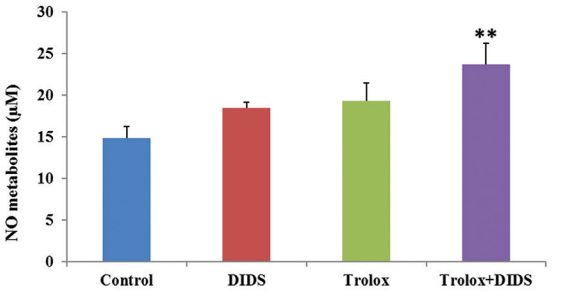

Figure 3: Concentrations of NO metabolites of the perfusate in Trolox+DIDS-treated was more than other groups at 30 minutes. Data are presented as mean±SE (n=5 in each group). **P<0.01 in Trolox+DIDS vs. control group.

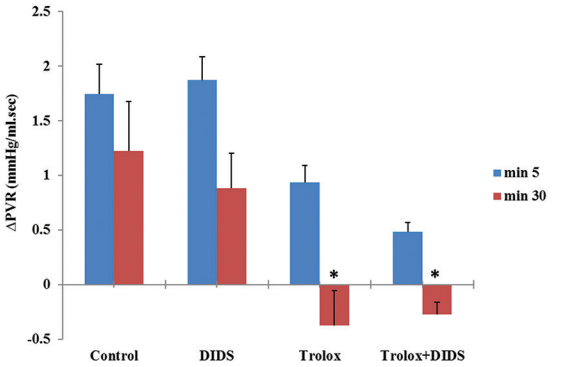

Figure 4: Pulmonary vascular resistance (ΔPVR) in Trolox-treated and Trolox+DIDS-treated groups at 30 minutes were lower than their values at 5 minutes. Data are presented as mean±SE (n=5 in each group). *P<0.05 at each group at 30 vs. 5 minutes.

was no variation between PVR in the Trolox and Trolox+DIDS groups (P=0.836) (figure 2).

The concentration of NO metabolites in the perfusate of the Trolox+DIDS group was significantly higher than the control group at 30 minutes (P=0.007). However, there was no significant difference between NO metabolites in the DIDS (P=0.169) and Trolox (P=0.101) groups compared to the control group (figure 3).

There was no alteration between PVR in the control group at 30 and 5 minutes (P=0.286). Furthermore, PVR in the DIDS group at 30 minutes decreased insignificantly compared to 5 minutes (P=0.184). However, PVR decreased significantly at 30 minutes compared to 5 minutes (P=0.038). Also, the reduction of PVR in the Trolox+DIDS group at 30 minutes was significant as compared with 5 minutes (P=0.013) (figure 4).

Discussion

Although HPV has been appreciated for about seven decades, but its mechanism is still controversial. So far, various mechanisms are attributed to this physiologic phenomenon, including endothelial derived substances and special characteristic of pulmonary smooth muscle cells.[7,26] It has been reported that ROS may increase or decrease during the exposure of pulmonary vessels to hypoxic gas, thus leading to HPV.[1,7] Furthermore, any changes in ROS

result in NO change in opposite direction.[20,21,27] Since there is no special carrier for O_2^- release from the pulmonary smooth muscle cells, it may exit through other transporters such as AE. The aim of this study was to investigate the interaction of O_2^- and AE in HPV. Our results indicate a possible role for AE in the acute phase of HPV. In addition, our findings indicate the effect of AE and ROS inhibition on NO production, as a factor for modulation of HPV.

PCO_2, HCO_3^- and pH in the perfusate did not alter throughout the experiments in the hypoxic groups, which confirm stable conditions over the time course of experiments. A similar reduction of PO_2 in all hypoxic groups verifies identical conditions of experiments between groups. We tested pulmonary vascular responses to hypoxic gas ventilation immediately after the steady-state period in order to examine the viability of the lungs and found no variation between the results in all groups (data not shown). Therefore, any alteration in hypoxic response of pulmonary vessels after the administration of drugs could be independent of the initial responses of the lungs. Furthermore, we calculated PVR in order to rule out the effect of the left atrial pressure and perfusate flow rate on pulmonary artery pressure. Ventilation of the lungs with hypoxic gas induced HPV being higher at 5 minutes and then started to reduce insignificantly until 30 minutes of the experiments. This data is comparable with data from previous studies showing a biphasic response of HPV in the isolated lung.[1,14,19]

The administration of Trolox decreased HPV at 5 minutes and entirely prevented it at 30 minutes. Trolox is a water-soluble analogue of vitamin E. It diffuses into both lipid and aqueous compartments and has a high potency for ROS scavenging.[28] The effects of Trolox in our experiments confirm the role of ROS during acute as well as the sustained phase of HPV, which is in line with previous studies.[5,13,19] However, the role of different ROS derivatives ($ONOO^-$, O_2^-, H_2O_2, and OH^-) on HPV cannot be differentiated by Trolox because of its wide spectrum antioxidant effects.[28]

The administration of DIDS had no further effect on HPV compared to the control group. The data is challenged by other studies postulating that the inhibition of AE or carbonic anhydrase decreases pulmonary vascular tone during hypercapnia.[12] Furthermore, 400 μM of DIDS extends the acute phase of HPV in the isolated rabbit lung whereas DIDS 200 μM has no effect on pulmonary artery pressure compared to the control hypoxic group during 60 minutes of the experiments.[15] Since DIDS is an inhibitor of AE and chloride channels, it can be assumed that the high doses of DID have more effect on HPV via inhibition of both anion transporters and channels. The expression of Na-dependent AE increases the pulmonary artery smooth muscle cell during hypoxia.[29] The activity of this exchanger leads to the influx of HCO_3^- into the cells in exchange for chloride anion. This may lead to intracellular alkalosis during HPV.[10,11] Inhibition of AE prevents HCO_3^- to enter the cells and lead to intracellular acidosis. However, there are conflicting results with regard to the effect of HPV on intracellular pH. Some researchers have reported intracellular alkalosis and a few studies have indicated intracellular acidosis during HPV.[11,30,31] It remains unclear whether changes in pH is a fundamental trigger for the induction of HPV.

The combination of Trolox and DIDS resulted in lower HPV compared with the effect of Trolox at 5 minutes even though it was not statistically significant. However, reduction of HPV in the Trolox+DIDS group compared with the control and DIDS groups was more than the Trolox group compared to the control and DIDS groups. O_2^- anion may exit from the pulmonary smooth muscle cells through AE in the isolated rat pulmonary artery and ischemia/reperfusion model in the isolated rabbit lung.[8,9,32,33] O_2^- release in exchange for HCO_3^- may lead to the enhancement of intracellular pH in the pulmonary smooth muscle cells. Furthermore, a part of O_2^- release may be related to chloride channels.[34] Overall, one could suggest that DIDS reduces HPV a fraction more than Trolox per se, perhaps by the prevention of intracellular alkalosis. It seems that the presence of ROS is fundamental for the induction of HPV and the activity of AE is necessary for alkalinization during HPV. However, additional studies with different doses of ROS inhibitors and AE inhibitors could specify the contribution of each in HPV.

NO metabolites in the perfusate were significantly higher in the Trolox+DIDS group compared to the control group. Inhibition of ROS increases NO production[20,21] and the inhibition of AE prevents ROS release from the pulmonary artery.[8] Each inhibitor insignificantly increased NO metabolites of the perfusate in the Trolox or DIDS group. The combination of Trolox and DIDS had additive effects on NO production and availability. NO is a vasodilator involved in the regulation of pulmonary vascular tone. More production in NO may lead to more reduction in HPV in the Trolox+DIDS group.

We did not measure extracellular and intracellular pH, and O_2^- due to certain limitations in our preparations. These measurements would

help us to confirm our hypothesis with regard to the possible interactions between ROS and AE. These issues require more investigation for further clarification.

Conclusion

In the present study, the interaction between ROS and AE in HPV has been assessed. A possible interaction is found by a mechanism partly linked to NO production and availability. However, future studies are required to discrete their contributions in this physiological phenomenon.

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Conflict of Interest: None declared.

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