Effects of oxidizing and reducing agents on ovine pulmonary artery responses to nitric oxide donors, sodium nitroprusside and 3-morpholino-sydnonimine

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Nitrovasodilators—sodium nitroprusside (SNP; 10^{-9}-10^{-4} M) and 3-morpholino-sydnonimine (SIN-1; 10^{-9}-10^{-4} M) produced concentration-dependent relaxation of the fourth generation sheep pulmonary artery, preconstricted with 5-hydroxytryptamine (1 µM). Oxidizing agents [oxidized glutathione (GSSG, 1 mM) and CuSO_{4} (5 and 20 µM)] and reducing agents [dithiothreitol (DTT, 0.1 mM), ascorbic acid (1 mM) and reduced glutathione (GSH, 1 mM)] caused opposite effects on nitric oxide (NO)-induced vasodilation in the artery. Ascorbic acid and GSH potentiated the NO responses, while GSSG and CuSO_{4} inhibited relaxation caused by the nitrovasodilators. DTT, however, reduced the relaxant potency and efficacy of SNP and SIN-1. Pretreatment of the pulmonary artery strips with DTT (0.1 mM) inhibited SNP (10 µM)-induced Na^{+}-K^{+}-ATPase activity, while ascorbic acid (1 mM) and GSH (1 mM) had no effect either on basal or SNP (10 µM)-stimulated \(^{86}\text{Rb}\) uptake, an index of Na^{+}-K^{+}-ATPase activity, in ovine pulmonary artery. The results suggest that reducing agents like ascorbic acid may have beneficial effect in improving the vascular function under oxidative stress.

Keywords: Nitric oxide, Ovine pulmonary artery, Redox regulation

Endogenous nitric oxide (NO), derived from vascular endothelium, produces effects through an indirect pathway involving activation of soluble guanylyl cyclase (sGC) and increased level of cyclic GMP. Oxidants and reductants are known to affect sGC activity\(^{1,2}\). For instance, thiol reductants, such as dithiothreitol (DTT) and reduced glutathione (GSH), have been found to have both inhibitory and stimulatory effects on sGC, while thiol oxidant, such as oxidized glutathione (GSSG) inhibits sGC activity, indicating the importance of redox processes in the regulation of sGC activity. Since both endogenous NO and NO donors primarily act through stimulation of sGC, it is important to examine the effects of redox-active agents on the vasodilator responses elicited by NO. In rat coronary artery, redox compounds have been shown to influence nitrovasodilator-induced relaxations \textit{in vitro}\(^^{3}\). Hence, in view of limited information on the cellular redox state in influencing vasodilation by different NO donors in pulmonary artery, the present study was undertaken to determine the influence of reducing and oxidizing compounds on vasodilation caused by sodium nitroprusside (SNP) and 3-morpholino-sydnonimine (SIN-1), using sheep pulmonary artery as a model.

Materials and Methods

Chemicals—Acetylcholine (ACh), 5-hydroxytryptamine (5-HT), dithiothreitol (DTT), sodium nitroprusside (SNP) and ouabain were procured from the Sigma Chemicals. GSSG and GSH were obtained from E. Merck. 3-Morpholino-sydnonimine (SIN-1), ascorbic acid and copper sulphate (CuSO_{4}) were procured from Tocris, BDH and SRL, respectively. All other chemicals used were of analytical grade.

Tissue—Lungs from freshly slaughtered adult sheep were collected from the local slaughterhouse within 20-30 min of slaughter in the modified Krebs-Henseleit physiological salt solution (4°-6°C), containing penicillin (100 units/ml) and streptomycin (100 µg/ml). The physiological solution contained (in mM, pH 7.4) NaCl, 118; KCl, 4.7; CaCl_{2}2H_{2}O, 2.5; MgSO_{4}.7H_{2}O, 1.2 NaHCO_{3}, 11.9; KH_{2}PO_{4}, 1.2 and D-glucose, 11.1 in triple distilled water. Intrapulmonary vascular network of the lung was exposed. The 4th generation intrapulmonary artery was dissected out and placed in physiological solution. After careful cleaning of adhering tissues, the artery was cut into rings of 3-4 mm length.

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Recording of isometric tension—The pulmonary arterial rings held in between two L-shaped hooks made of 30-gauge stainless steel wire were mounted in a thermostatically controlled (37±0.5°C) organ bath of 20 ml capacity, containing physiological solution that was continuously aerated with carbogen (95% O₂ and 5% CO₂). The tissue was equilibrated for 1.5 hr under a resting tension of 1 g. During the equilibration, the bathing fluid was changed every 15 min. The change in tension was measured with an isometric force transducer (Model: MLT 0202/D, Powerlab, Australia) and recorded in a computer, using Chart V4.1.2 software (Powerlab, Australia).

Redox regulation in endothelium-denuded ovine pulmonary artery—The arterial rings were primed with 5-HT (1 µM) and when the contraction attained plateau, ACh (100 µM) was added to determine the endothelial integrity. A contractant or relaxant response to ACh confirmed absence or presence of functional endothelium. The tissue was washed with physiological solution to restore the baseline tension. The arterial rings were then contracted submaximally with 5-HT (1 µM) and when the contraction was stable, nitrovasodilator SNP (10⁻⁹-10⁻⁴ M) or SIN-1 (10⁻⁹-10⁻⁴ M) was added cumulatively at an increment of 1 log unit until maximal reversal of 5-HT-induced contraction was obtained. After several washes with physiological solution, the tissues were then exposed to the individual reducing or the oxidizing agent for 30 min before the second concentration-response curve was elicited with SNP (10⁻⁹-10⁻⁴ M) or SIN-1 (10⁻⁹-10⁻⁴ M). The reducing agents used were DTT (0.1 mM), ascorbic acid (1 mM) and GSH (1 mM), while the oxidizing agents were GSSG (1 mM) and CuSO₄ (5 and 20 µM). The effects of individual reducing or oxidizing agent on basal tension, 5-HT-induced contraction and vasodilatory potency and efficacy of SNP or SIN-1 were evaluated.

Estimation of Na⁺-K⁺-ATPase activity—Isolation of sarcolemmal membranes from ovine pulmonary arteries was done following the procedure of Matlab et al. Na⁺-K⁺-ATPase activity was determined by measuring the liberation of inorganic phosphate (Pi) from ATP in 1 ml of medium containing (mM) Tris HCl buffer, 50, pH 7.5; NaCl, 140; KCl, 14; MgCl₂, 6H₂O, 5; EDTA, 0.5; ouabain, 1 and requisite volume of membrane homogenate (10 µl) in a final volume of 1 ml. The reaction mixture was preincubated for 5 min at 37°C. The reaction was started with the addition of 3 mM ATP. For total ATPase assay, ouabain was omitted from the reaction mixture, which was included for Mg²⁺-ATPase assay. After 1 hr of incubation at 37°C in both cases, the reaction was stopped by adding 0.1 ml of cold 5% sodium dodecyl sulphate, and colour was developed with 3 ml of acidic ammonium molybdate and 0.1 ml of ANSA reagent (1-Amino-2 naphthol-4-sulfonic acid, 25 mg; sodium metabisulphite, 1.2 g; sodium sulphite, 120 mg dissolved in 10 ml of distilled water). The inorganic phosphate in the reaction mixture was assayed according to the method of Yohtalou. A standard phosphate (10 µg/ml) and blank were run simultaneously. The difference in the activity in the absence and presence of ouabain was taken as Na⁺-K⁺-ATPase activity. Protein content in the membrane fraction was determined by Lowry’s method. Specific enzyme activity has been expressed as nmol of Pi liberated/min/mg of protein. To determine the effect of DTT on SNP-induced Na⁺-K⁺-ATPase activity, tissues were pretreated with SNP (10 µM) or SNP plus DTT (0.1 mM) for 30 min and then the Na⁺-K⁺-ATPase activity was determined as described above. Basal activity of the enzyme was measured following incubation of the tissues for 30 min in physiological solution.

Measurement of ouabain-sensitive ⁸⁶Rb uptake—⁸⁶Rb uptake by pulmonary artery was determined following the method of Gupta et al. Arterial rings were equilibrated in physiological solution for 1.5 hr at 37°C and aerated continuously with carbogen. After the equilibration, the test drugs were added for 30 min followed by ouabain (0.2 mM) for 10 min. Then ⁸⁶RbCl (2 µCi/ml) was added into the incubating vials for 10 min. The tissues were washed in cold (4°C) unlabelled physiological solution for 2 min to remove the radioisotope from the extracellular compartments, blotted on the filter paper and dried overnight in an oven maintained at 100°C. ⁸⁶Rb content of the tissue was determined by gamma counting. Ouabain-sensitive ⁸⁶Rb uptake, which is known to be an index of Na⁺-K⁺-ATPase activity, was calculated by subtracting ⁸⁶Rb uptake in the presence of maximally effective concentration of ouabain (0.2 mM) from total ⁸⁶Rb uptake.

Statistical analysis—Relaxation responses have been expressed as a percentage reversal of the serotonin-induced precontraction. Results have been expressed as mean ± SE with n equal to number of vascular rings. EC_{50} concentrations were calculated.
by linear regression analysis and expressed as negative log molar concentration of the agonist (pD₂), i.e., \(-\log [B]\), where [B] is the molar concentration of the agonist, which produces half-maximal response. Non-linear regression with sigmoidal curve fitting was used to determine E_max. Data on relaxation responses were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test, while on Na⁺-K⁺-ATPase activity and ⁸⁶ rubidium uptake by one-way ANOVA followed by Tukey's post-hoc test. Difference between the means at \( P < 0.05 \) was considered statistically significant.

**Results**

**Effect of time and vehicle on 5-HT-induced sustained contraction**—Figures 1A and 1B show the effects of time/vehicle and SNP on 5-HT-precontracted ovine pulmonary artery, respectively. Time and vehicle had no significant effect on the plateau phase of 5-HT-induced contraction.

**Effects different treatments on absolute tension generated by 5-HT**—Effects of SNP or SIN-1 alone and following preincubation with reducing or oxidizing agent are presented in Table 1. Preincubation of the tissue with DTT (0.1 mM), ascorbic acid (1 mM), GSH (1 mM), or GSSG (0.1 mM) had no significant effect on 5-HT-induced contraction. Preincubation of the arterial rings with CuSO₄ (5 and 20 µM) increased the basal tone by 14 %, but failed to alter the 5-HT-induced contraction. Thus, the absolute tension produced by 5-HT in presence of individual reducing or oxidizing agent was comparable to that of the respective control one.

**Effect of DTT on SNP- and SIN-1-induced responses in ovine pulmonary artery**—SNP (10⁻⁸-10⁻⁴ M) or SIN-1 (10⁻⁷-10⁻³ M) produced concentration-dependent relaxation of ovine pulmonary artery precontracted with 5-HT (1 µM) (Figs 2A and 2B). The pD₂ and E_max values of SNP were 6.42 ± 0.04 and 97.49 ± 1.74% and of SIN-1 were 6.39 ± 0.07 and 99.21 ± 1.16%, respectively. DTT caused a rightward shift in the concentration-response curve of SNP or SIN-1 with significant decrease in the pD₂ and E_max to 5.65 ± 0.07 and 78.79 ± 1.85% or 5.62± 0.19 and 80.44 ± 1.92%, respectively.

**Effect of ascorbic acid on SNP- and SIN-1-induced responses in ovine pulmonary artery**—SNP (10⁻⁹-10⁻⁴ M) or SIN-1 (10⁻⁹-10⁻⁴ M) produced concentration-related relaxation in 5-HT (1 µM)-mediated precontracted pulmonary arterial rings (Figs 3A and 3B). The respective pD₂ and E_max values of SNP were 6.17 ± 0.06 and 93.25 ± 0.71% while of SIN-1 were 6.04 ± 0.05 and 93.69 ± 1.17%. Unlike DTT, ascorbic acid caused a leftward shift in the concentration-response curve of SNP or SIN-1 with significant increase in the pD₂ and E_max to 6.53 ± 0.06 and 104.82±1.97% or 6.48±0.04 and 103.47±1.06%, respectively.

![Fig. 1](image_url)—Original tracings of contractions showing the effects of (A) time/vehicle (physiological salt solution) and (B) sodium nitroprusside (SNP) on 5-hydroxytryptamine (5-HT)-precontracted ovine pulmonary artery.

<table>
<thead>
<tr>
<th>Reductant/oxidant</th>
<th>5-HT (1 µM)-induced contraction (g)</th>
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<tbody>
<tr>
<td>SNP (10⁻⁶ M)</td>
<td>Control 0.91 ± 0.10 (6) 0.89 ± 0.06 (6)</td>
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<tr>
<td></td>
<td>Dithiothreitol 0.97 ± 0.16 (6) 0.82 ± 0.05 (6)</td>
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<td></td>
<td>Control 0.89 ± 0.09 (6) 0.88 ± 0.14 (6)</td>
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<td></td>
<td>Ascorbic acid 0.75 ± 0.10 (6) 0.81 ± 0.07 (6)</td>
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<td></td>
<td>Control 0.80 ± 0.13 (6) 1.21 ± 0.13 (6)</td>
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<td></td>
<td>GSH (1 mM) 0.79 ± 0.13 (6) 1.26 ± 0.14 (6)</td>
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<tr>
<td></td>
<td>Control 0.57 ± 0.05 (6) 1.64 ± 0.15 (6)</td>
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<td></td>
<td>GSSG (0.1 mM) 0.63 ± 0.05 (6) 1.57 ± 0.21 (6)</td>
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<td></td>
<td>Control 0.77 ± 0.08 (11) 1.23 ± 0.25 (6)</td>
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<tr>
<td></td>
<td>CuSO₄ (5 µM) 0.64 ± 0.06 (6) 1.26 ± 0.20 (6)</td>
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<td>CuSO₄ (20 µM) 0.77 ± 0.09 (5) –</td>
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SNP = Sodium nitroprusside; SIN-1 = 3-morpholino-sydnonimine
Effect of GSH on SNP- and SIN-1-induced responses in ovine pulmonary artery—In the precontracted (5-HT, 1 µM) pulmonary arterial rings, both SNP (10^{-9}-10^{-4} M) or SIN-1 (10^{-9}-10^{-4} M) elicited concentration-dependent relaxation (Figs 3C and 3D). The pD2 and E_{max} values of SNP were 5.85 ± 0.08 and 80.83 ± 1.37% and of SIN-1 were 5.95 ± 0.08 and 80.97 ± 2.09%, respectively. Similar to ascorbic acid, GSH caused a leftward shift in the concentration-response curve of SNP or SIN-1 with significant increase in the respective pD2 and E_{max} to 6.24 ± 0.05 and 89.26 ± 1.49% or 6.42 ± 0.01 and 92.95 ± 1.60%.

Effect of GSSG on SNP- and SIN-1-induced responses in ovine pulmonary artery—SNP (10^{-9}-10^{-4} M) or SIN-1 (10^{-9}-10^{-4} M) caused concentration-related relaxation in 5-HT (1µM)-mediated precontracted pulmonary arterial rings (Figs 4A and 4B). The pD2 and E_{max} values of SNP were 5.94±0.02 and 82.81 ± 1.58% while of SIN-1 were 6.01 ± 0.07 and 83.77 ± 1.57%, respectively. GSSG inhibited the vasodilator responses of SNP and SIN-1 and caused a rightward shift in the concentration-response curve with significant decrease in the pD2 and E_{max} to 4.44 ± 0.10 and 51.98 ± 1.63% or 5.08± 0.10 and 64.79 ± 1.76%, respectively.

Effect of CuSO4 on SNP- and SIN-1-induced responses in ovine pulmonary artery—In the
precontracted (5-HT, 1 μM) pulmonary arterial rings, SNP (10⁻⁹-10⁻⁴ M) or SIN-1 (10⁻²-10⁻⁴ M) elicited concentration-dependent relaxation (Figs 4C and 4D). The respective pD₂ and E_max values of SNP were 5.81 ± 0.10 and 80.67±1.03% while of SIN-1 were 6.07±0.07 and 84.47±1.19%. CuSO₄ (5 and 20 μM) markedly inhibited the vasodilator responses of SNP and caused a rightward shift in the concentration-response curve of SNP with a significant decrease in the E_max to 42.13±1.89% or 31.70±1.69%, respectively. CuSO₄ (5 μM) also caused a marked inhibition in the vasodilatory effect of SIN-1 with a significant decrease in the E_max to 36.97 ± 1.19%. In both the cases, the pD₂ values could not be determined as the maximal responses were lesser than 50%.

**Effect of reducing agents on SNP-induced Na⁺-K⁺-ATPase activity and ouabain-sensitive ⁸⁶Rb uptake**—Pretreatment of the pulmonary artery strips with DTT (0.1 mM) for 30 min significantly inhibited the SNP (10 μM)-induced Na⁺-K⁺-ATPase activity (Fig. 5). Ascorbic acid (1 mM) and GSH (1 mM) had no significant effect either on basal or SNP (10 μM)-stimulated ⁸⁶Rb uptake in ovine pulmonary artery (Fig. 6).

**Discussion**

In the present investigation, five redox compounds were used to study their effects on nitrovasodilator-induced relaxation in ovine pulmonary artery. Amongst them, ascorbic acid, an endogenous reductant; DTT, a dithiolreductant; and GSH, a thiolreductant, were employed as reducing agents, while CuSO₄ and GSSG were used as oxidants to study their influence on the relaxation mediated by NO donors – SNP and SIN-1. The major observation of the present study is that oxidizing and reducing agents produced opposite effects on NO-induced relaxation of vascular smooth muscle in ovine pulmonary artery. While reducing agents, ascorbic acid and GSH, potentiated the NO-responses; CuSO₄ and GSSG consistently inhibited relaxation caused either by endogenous NO or NO donors (SNP and SIN-1). DTT, however, reduced the relaxant potency and efficacy of SNP and SIN-1 in ovine pulmonary artery.

Ascorbic acid and GSH potentiated the relaxant responses of NO donors, SNP and SIN-1 in ovine pulmonary artery. Reductants are known to optimize sGC activity. However, the inability of DTT to potentiate the responses to SNP and SIN-1 goes against this possibility. There are reports suggesting a decrease in NO/NO donor relaxant responses by DTT. Consistent with the previous findings, it was observed that DTT inhibited the vasodilator response to SNP and SIN-1. It is known that reductants like DTT accelerate the release of NO from nitroprusside. One possibility is that DTT accelerates breakdown, but the NO released is
immediately consumed in a reaction with superoxide, so that the net effect is usually a lower concentration of nitroprusside in solution. Plasma membrane Na\(^+\)-K\(^+\)-ATPase has been identified as a primary target for SNP-induced dilation of ovine pulmonary artery. In the present study, it was observed that DTT, a sulfhydryl reagent, partially inhibited stimulation of Na\(^+\)-K\(^+\)-ATPase activity by SNP. Sweadner indicated that sulfhydryl reagents like N-ethylmaleimide inhibit Na\(^+\)-K\(^+\)-ATPase activity in brain and kidney. It is, therefore, possible that DTT through this mechanism may attenuate NO-mediated relaxation of ovine pulmonary artery.

Effects of ascorbic acid on NO/nitrovasodilator-mediated relaxation on different blood vessels are variable. For example, ascorbic acid reduced the responses to endothelium-derived nitric oxide (EDNO) and authentic NO in rabbit aortic rings. In the same preparation, it potentiated relaxation to nitroprusside and S-nitroso-N-acetyl penicillamine (SNAP), but again inhibited relaxation to glyceryl trinitrate. In rat coronary artery, ascorbic acid potentiated the relaxant responses to SIN-1 and SNAP. Potentiation of the responses to SNP and SIN-1 by ascorbic acid and GSH in ovine pulmonary artery possibly relates to a common mechanism of potentiation by these two reducing agents. It is believed that amongst the three different redox species of NO [viz., nitroxy anion (NO\(^-\)), nitrosonium cation (NO\(^+\)) and nitric oxide radical (NO\(^\bullet\))], the NO\(^-\) is the most physiologically relevant, as it is produced in abundance endogenously. It is possible that NO\(^-\) derived from SNP and NO\(^-\) released from SIN-1 undergo reduction in presence of reducing agents to form NO\(^\bullet\). This possibly is the pathway of potentiation of the nitrovasodilator responses by the reducing agents. Effect of ascorbic acid was assessed on \(^{86}\)Rb-uptake in ovine pulmonary artery so as to determine the contribution of Na\(^+\)-K\(^+\)-ATPase to the potentiation of NO responses by the reducing agent. The results showed that ascorbic acid had no significant effect either on basal or SNP-stimulated \(^{86}\)Rb-uptake in ovine pulmonary artery. Therefore, the contribution of Na\(^+\)-K\(^+\)-ATPase to ascorbic acid-induced potentiation of NO responses is ruled out. Similar to ascorbic acid, GSH also neither significantly altered the basal nor SNP-stimulated \(^{86}\)Rb-uptake in ovine pulmonary artery.

The present results with ascorbic acid are consistent with the previous reports, wherein this compound was shown to improve endothelium-dependent flow-mediated dilation in patients with coronary artery disease and lowering of pressure in hypertensive subject. Inactivation of EDNO by reactive oxygen species (ROS), viz., \(\text{O}_2^−\) may inactivate NO to produce vascular dysfunction. The observations of the present study with ascorbic acid suggest that the antioxidant may improve pulmonary circulation in the event of an oxidative stress.

Oxidizing agents, viz., CuSO\(_4\) and GSSG may reduce the availability of NO species thereby inhibiting relaxant responses of SNP and SIN-1 in ovine pulmonary artery. With respect to CuSO\(_4\), the present results are at variance with the finding obtained in rabbit aorta by De Saram et al. who reported that CuSO\(_4\) had no significant effect on relaxation to nitroprusside in rabbit aortic rings. CuSO\(_4\) has, however, been shown to reduce ACh-induced relaxation in rat aorta. Copper has been shown to increase tissue cyclic GMP level in rat pulmonary artery and to stimulate NO synthase activity. These mechanisms, however, do not explain the inhibitory effect we observed in ovine pulmonary artery. It is quite possible that oxidizing agents, viz., GSSG and CuSO\(_4\) may reduce the availability of NO\(^-\) species for the vasodilator responses of SNP and SIN-1 in ovine pulmonary artery. The other possibility of copper inhibiting nitrovasodilator responses may relate to \(\text{O}_2^\bullet\) generation by this transition metal, which reacts with NO to produce peroxynitrite. Endothelial production of \(\text{O}_2^\bullet\) by copper has been reported to cause altered NO activity and endothelial dysfunction.

In conclusion, oxidizing agents have inhibitory effect, while the reducing agents have an opposite effect on the vasorelaxation mediated by NO donors – SNP and SIN-1 in ovine pulmonary artery. Further studies are required to define their mechanisms of action. However, the observed finding with ascorbic acid in ovine pulmonary artery suggests that the antioxidant may improve pulmonary circulation in the event of an oxidative stress.

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References


