Experimental Physiology

Superoxide dismutase mimetic tempol inhibits hypoxic pulmonary vasoconstriction in rats independently of nitric oxide production

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Hypoxic pulmonary vasoconstriction (HPV), an important physiological mechanism, is regulated by changes in the production of and interactions among reactive oxygen species (ROS). There is controversy, however, over whether HPV is mediated by an increase or a decrease in ROS production. Also, the role of NO in HPV remains unclear. The aim of this study was to investigate whether the inhibition of HPV by the antioxidant tempol was dependent on the concentration of NO, and how its effect was influenced by increased basal pulmonary vascular tone. In isolated rat lungs, we measured vasoconstrictor responses to acute ventilatory hypoxia before and after administration of tempol during perfusion with or without L-NAME. We found that tempol abolished HPV independently of NO production. When we increased basal vascular tone by K^+-induced depolarization, we also found that tempol completely inhibited HPV. Our results indicate that inhibition of HPV by the superoxide dismutase mimetic tempol does not depend on either NO production or a decrease in basal vascular tone.

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Hypoxic pulmonary vasoconstriction (HPV) reduces blood flow to poorly ventilated areas of the lung in favour of perfusion of better ventilated regions. It optimizes lung ventilation/perfusion ratio and consequently also the oxygenation of arterial blood. As a local regulatory mechanism, HPV is fast in onset and readily reversible upon reoxygenation.

The presence and intensity of HPV depends on interactions between reactive oxygen species (ROS) and NO produced in pulmonary arteries. It has been demonstrated that acute ventilatory hypoxia causes changes in production of superoxide via mitochondrial electron transport chain or NADPH oxidase activity (Marshall *et al.* 1996; Liu *et al.* 2006). Administration of superoxide dismutase (SOD) or of a non-selective NADPH oxidase inhibitor, diphenyleneiodonium, significantly attenuated HPV (Thompson *et al.* 1998; Liu *et al.* 2003). Lung hypoxia stimulates NO production, and inhibitors of NO synthesis potentiate HPV (for review see Hampl & Herget, 2000). In the presence of NO, superoxide anion rapidly forms peroxynitrite, which also causes pulmonary vasoconstriction (Belik *et al.* 2004). This reaction is almost 10 times faster than the removal of superoxide anion by SOD. As a result of this, superoxide has a direct effect on NO concentration. Therefore, scavenging superoxide may enhance NO activity and prevent peroxynitrite formation, leading to inhibition of HPV. In contrast, superoxide scavenging might have a direct effect on HPV by reducing the concentration of a mediator reactive oxygen species.

Thus, the purpose of this study was to test, in isolated, physiological saline-perfused lungs, whether HPV is modulated mainly by superoxide production, independently of NO synthesis, or by a product of superoxide–NO interaction. For this purpose, we used the intracellularly acting ROS scavenger tempol (4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl) and the NO synthesis inhibitor L-NAME.

The vasoconstrictor response to acute ventilatory hypoxia is dependent on previous stimulation. In lungs repeatedly challenged with a particular hypoxic gas mixture, the hypoxic pressor response tends to increase (Archer *et al.* 1989*a*). Higher vasoconstrictor responses can also be achieved by prior increase in basal vascular tone (McMurtry, 1984). In the second part of this study, we tested whether the inhibitory effect of tempol was dependent on basal pulmonary vascular tone.

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Methods

Experiments were performed on 27 adult male rats (age 7–8 weeks, average weight 240 ± 15 g) in accordance with the European Community and US National Institutes of Health guidelines for using experimental animals. All procedures were approved by the Animal Studies Committee in the authors' institution.

Isolated lungs

Isolated, physiological saline-perfused lungs were prepared as previously described (Herget *et al.* 1987; Hampl & Herget, 1990; Hampl *et al.* 1993). The rats were anaesthetized with an intraperitoneal injection of sodium thiopentone (40 mg (kg body weight)$^{-1}$) and ventilated with air through a tracheal cannula (50 breaths min^{-1}; peak inspiratory pressure 10 cmH_20, positive end-expiratory pressure 2 cmH_20). The chest was then opened and the main pulmonary artery and left atrium were cannulated. Using a peristaltic pump, the lungs were perfused with physiological salt solution with albumin (4 g (100 ml)$^{-1}$; Herget & McMurtry, 1985) and meclofenamate (1.7×10^{-5} M) at a constant flow rate (4 ml min^{-1} (100 g)$^{-1}$). While being ventilated with normoxic gas mixture (21% O_2–5% CO_2–74% N_2), the heart–lung block was excised from the body and suspended by the trachea in a heated (38°C) humidified chamber. The outflow cannula from the left atrium was connected into the circuit via the perfusate reservoir after the outflow was free of blood. The outflow pressure was set to –2 mmHg. The pulmonary perfusion pressure was measured via a transducer connected to the inflow cannula (PowerLab, AD Instruments, Spechbach, Germany). Owing to the constant-flow perfusion mode, the values of measured perfusion pressure corresponded to pulmonary vascular resistance.

Experimental protocol

Experiment A: effect of ROS and NO inhibition on HPV. The isolated lung was allowed to stabilize for 15 min before the vascular reactivity in response to angiotensin II (ATII; 0.2 μg) and to acute hypoxia (0% O_2–5% CO_2–95% N_2) was tested. The bolus of ATII injected into the inflow line caused a short (less than 2 min) transient rise in perfusion pressure. Six minutes of acute hypoxia was started 8 min after the ATII injection. This ATII–hypoxia cycle was performed twice, with 10 min intervals between cycles. Physiological saline-perfused lungs are hyporeactive relative to preparations perfused with blood or plasma, so two ATII–hypoxia challenges are usually used to induce comparable pressor reactivity (Fishman, 1976; McMurtry, 1984; Herget & McMurtry, 1987). Ten minutes after the second hypoxic challenge, tempol was added to the perfusate at a dose of 0.5 mg (ml perfusate)$^{-1}$. In our preliminary experiments, after this dose we observed 81% inhibition of response to ATII and 67% inhibition of response to acute hypoxia. This dose also corresponds to the recommended dose of 50 mg kg^{-1} of tempol (Thiemermann, 2003). After 10 min of equilibration, two ATII–hypoxia cycles were performed (control group, $n = 6$). In the second experimental group (L-NAME group, $n = 6$), the production of NO was inhibited by addition of the non-selective inhibitor of all NO synthases, L-NAME (5×10^{-5} M), to the perfusate at the start of perfusion. We then repeated the same experimental protocol as described for the control group. Changes in perfusion pressure caused by the second and fourth ATII–hypoxia cycles were compared (Figs 1 and 2).

Experiment B: effect of increase of basal vascular tone on tempol-induced inhibition of HPV. Since we know that the vasoconstrictor response to acute ventilatory hypoxia is dependent on previous stimulation, as well as on the level of basal vascular tone (Fishman, 1976; McMurtry, 1984), we decided to investigate whether the tempol-induced inhibition of HPV is also dependent on basal vascular tone. To increase basal vascular tone in the perfused lungs, we evoked smooth muscle depolarization by increasing the potassium ion concentration in the perfusate by 15 mmol l^{-1} (final K^+ concentration of 19.7 mmol l^{-1}).

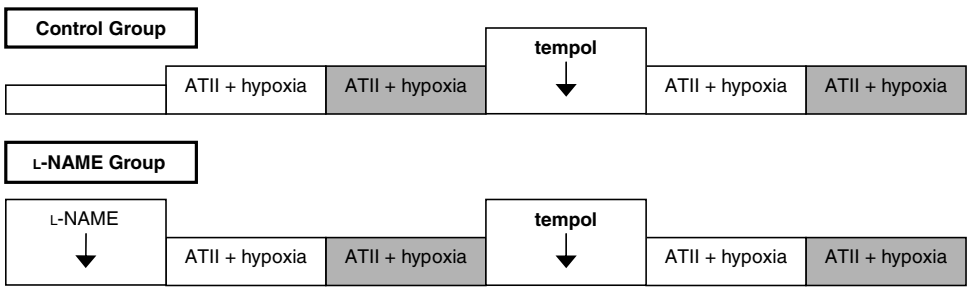

Figure 1. Experimental protocol of experiment A: effect of inhibition of ROS and NO production on HPV
'Hypoxia' represents a 6 min ventilation with a gas mixture of 0% O_2–5% CO_2–95% N_2. 'ATII' represents a bolus intra-arterial injection of angiotensin II (0.2 μg). We compared the second and fourth response to ATII and hypoxia (shaded areas).

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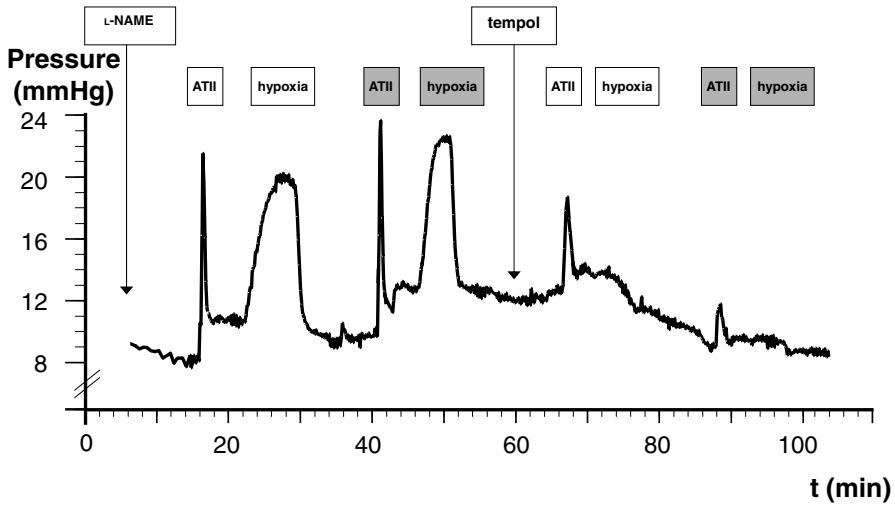

Figure 2. Original record of the measurement of perfusion pressure in experiment A

At the start of perfusion in the K^+ + tempol group ($n = 5$) and the K^+ group ($n = 5$), we added KCl into the perfusate to increase K^+ concentration by 15 mmol l^{-1}. After 15 min of equilibration, the lungs were exposed to two successive hypoxic challenges and then stimulated by bolus injection of ATII followed by two additional hypoxic challenges. Then in the tempol group ($n = 5$) and the K^+ + tempol group, tempol was administered into the perfusate at a dose of 0.5 mg ml^{-1}. Thereafter we performed two further hypoxic challenges. We compared the changes in perfusion pressure caused by second, fourth and sixth challenge of acute ventilatory hypoxia (Fig. 3).

Statistical analyses

The results are presented as means ± S.E.M. They were statistically evaluated by Student's paired *t* test comparison or ANOVA and Fisher's PLSD test, as appropriate. The statistical analyses were performed using the statistical software StatView 5.0 (SAS Institute Inc., Cary, NC, USA). Differences were considered significant at $P < 0.05$.

Results

Experiment A

In both the control and L-NAME groups, we found significant inhibition of vasoconstrictor responses to both ATII and hypoxia after administration of tempol. Tempol inhibited 86% of the vasoconstrictor response to ATII in the control and 83% in the L-NAME group. Hypoxia-induced vasoconstriction was 89% less in the control and 100% less in the L-NAME group.

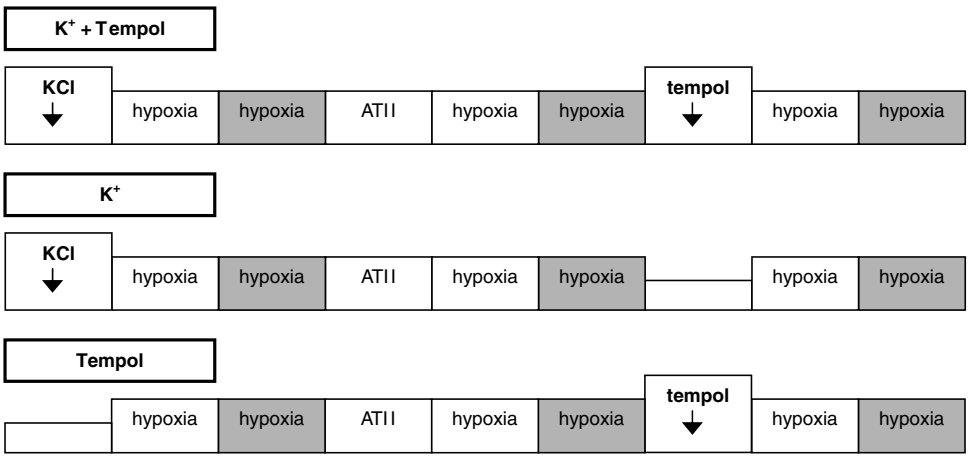

Figure 3. Experimental protocol of experiment B: effect of increase in basal vascular tone on tempol-mediated inhibition of HPV

'Hypoxia' represents 6 min ventilation with a gas mixture of 0% O_2–5% CO_2–95% N_2. 'ATII' represents a bolus intra-arterial injection of angiotensin II (0.2 μg). 'K^+' represents 15 mmol l^{-1} K^+ ions added to the perfusate. The magnitudes of the second, fourth and sixth hypoxic challenges were compared (shaded areas).

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Tempol-mediated inhibition of hypoxic vasoconstriction did not differ when comparing the two hypoxic challenges after administration of tempol (third and fourth hypoxia: control group 0.4 ± 0.1 *versus* 0.6 ± 0.2 mmHg, $P = 0.33$; L-NAME group 0.5 ± 0.2 *versus* -0.1 ± 0.2 mmHg, n.s.). Comparison of tempol-induced inhibition of vascular responses to acute hypoxic challenge in control and L-NAME groups (control group + tempol *versus* L-NAME group + tempol) did not reveal any significant difference. This suggests that NO production does not mediate the tempol-induced inhibition of HPV (Figs 4 and 5).

Experiment B

Addition of KCl into the perfusate and the subsequent increase in K^+ concentration in the K^+ + tempol group and K^+ group increased basal perfusion pressure (K^+ + tempol group from 9.8 ± 0.8 to 12.5 ± 1.6 mmHg, $P = 0.024$; K^+ group: from 10.5 ± 0.6 to 13.5 ± 1.1 mmHg, $P = 0.008$). There was no difference in the increased pressure between these two groups. Vascular responses to ventilatory hypoxia (second response in Fig. 3) were higher when the concentration of K^+ ions in the perfusate was increased. We observed significantly higher ($P < 0.05$) hypoxic pulmonary vasoconstriction in the K^+ + tempol group and the K^+ group compared with HPV in the tempol group. Looking at hypoxic responses after ATII administration (fourth response in Fig. 3), we did not find any differences between the groups.

Administration of tempol (K^+ + tempol group and tempol group) significantly decreased hypoxic pulmonary vasoconstriction in both the K^+-treated (96% less) and in K^+-untreated group (68% less), and the vasoconstrictor response did not differ significantly between these groups. In contrast, the intensity of HPV in the tempol-untreated group (K^+ group) was constant, attesting to the viability of the preparation over the duration of the protocol (Fig. 6). When comparing the two hypoxic challenges after tempol administration (fifth and sixth period of hypoxia), we did not find statistical differences in any of the groups (K^+ + tempol group 0.2 ± 0.0 *versus* 0.1 ± 0.0 mmHg, n.s.; K^+ group 2.8 ± 0.5 *versus* 2.7 ± 0.4 mmHg, n.s.; tempol group 0.8 ± 0.2 *versus* 0.8 ± 0.1 mmHg, n.s.).

Discussion

The main findings of this study are as follows.

(1) Superoxide production plays a role in HPV. Its dismutation into hydrogen peroxide by the superoxide dismutase mimetic tempol significantly attenuates vasoconstrictor responses to acute ventilatory hypoxia.

(2) The effect of superoxide dismutation is directly related to a decrease in superoxide or increase in hydrogen peroxide concentration. It is not caused by overproduction of NO.

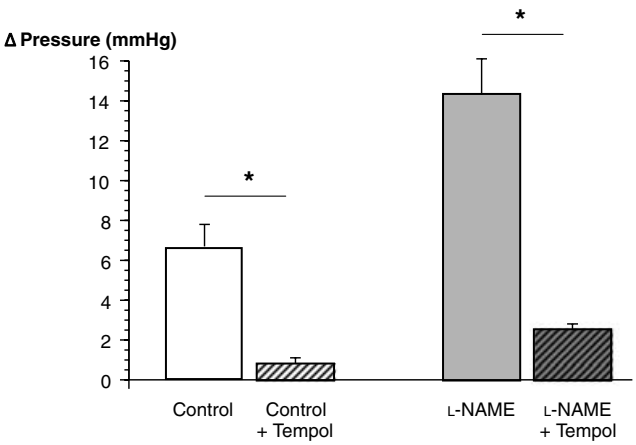

Figure 4. Effect of tempol and tempol + L-NAME on ATII-induced vasoconstriction
The figure shows the difference in the response to ATII before (open and shaded columns) and after administration of tempol (hatched and shaded hatched columns). After tempol administration, we found a significant decrease of ATII-induced vasoconstriction in both the control and the L-NAME group. There was no difference in this respect between the groups. ΔP represents the change in vascular tone induced by administration of ATII; $^*P < 0.005$. Columns show data for the control and L-NAME groups before (second ATII–hypoxia cycle) and after administration of tempol (fourth ATII–hypoxia cycle).

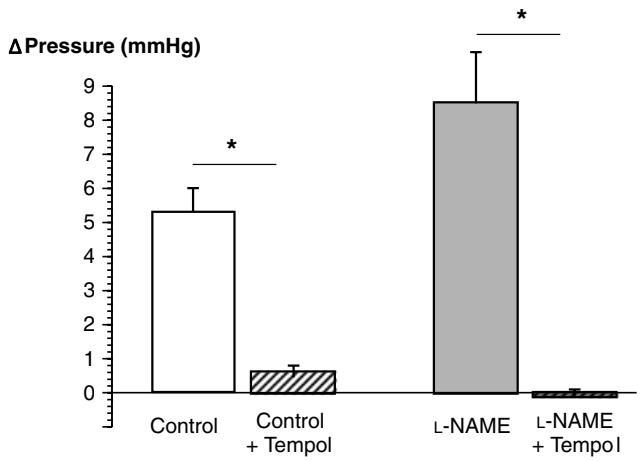

Figure 5. Effect of tempol and tempol + L-NAME on HPV
The figure shows the difference in the response to hypoxia before (open and shaded columns) and after administration of tempol (hatched and shaded hatched columns). After tempol administration, we found a significant decrease of HPV in both the control and the L-NAME group. Just as after angiotensin administration, in HPV we did not find any difference between the control and L-NAME group. ΔP represents the change in vascular tone induced by acute hypoxia; $^*P < 0.005$. Columns show data for the control and L-NAME groups before (second ATII–hypoxia cycle) and after administration of tempol (fourth ATII–hypoxia cycle).

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(3) The reduction of HPV in the presence of tempol does not depend on the basal tone of the pulmonary vessels.

Although there is considerable evidence that ROS play an important role in the regulation of pulmonary vascular tone during acute and chronic ventilatory hypoxia, the mechanism, source and reaction pathways involved in the regulation remain unclear. In addition to the controversy over whether the source of ROS is in the mitochondrial electron transport chain (Waypa *et al.* 2001) or in NADH oxidase (Gupte *et al.* 2005), another important debate concerns the character of ROS concentration change during hypoxia.

One group of investigators presents the view that HPV is evoked by sudden decrease in ROS production. The redox theory of HPV, developed by Archer *et al.* (1986), is based on the assumption of continual, tonic production of ROS during normoxia. They observed rapid inhibition of radical production both in isolated perfused lungs and in isolated pulmonary artery rings during acute hypoxia. In the proposed mechanism, the electron transport chain is altered by hypoxia, and ROS production decreases. Reduction or oxidation of cysteine and methionine groups by a redox mediator, such as ROS, can cause conformational changes in the K_V channels. This K_V channel inhibition depolarizes the membrane and activates Ca^{2+} entry via L-type Ca^{2+} channels (Moudgil *et al.* 2005). According to this theory, there is definitely a physiological role for continual ROS production. Superoxide is produced at very low levels during normoxia, and antioxidant enzymes such as SOD reduce superoxide to H_2O_2, which is subsequently reduced by catalase to H_2O and O_2. This mechanism serves both in cellular protection against the potentially damaging effects of superoxide and for continual regulatory production of H_2O_2. A fall in H_2O_2 concentration leads to HPV. Although Wolin's group believe that NADH oxidase, instead of the mitochondrial electron transport chain, is the relevant source of ROS, they have also found that a decrease of H_2O_2 production elicits HPV (Gupte *et al.* 2005).

In contrast, Liu *et al.* (2003) measured ROS production using dichlorofluorescein fluorescence, lucigenin-derived chemiluminescence (LDCL) and electron paramagnetic resonance (EPR) spin adduct spectra in small distal porcine pulmonary arteries. They found that radical production was increased during hypoxia. The same conclusions result from observations made by Schumacker's group, who also found an increase of ROS production evoked by acute hypoxia (Waypa *et al.* 2001; Waypa & Schumacker, 2005).

In our study, we have used tempol to decrease the superoxide concentration. Tempol permeates biological membranes and works intracellularly (Thiemermann, 2003). It has been reported that the protective effects of tempol result from its ability to scavenge superoxide anions and hydroxyl radicals (Thiemermann, 2003; Sainz *et al.* 2005). In activated neutrophils, tempol does not decrease H_2O_2 and the protective effect cannot be attributed to catalase-like activity (Hahn *et al.* 1997). Since we have observed that tempol inhibits HPV, we assume that superoxide is reduced to H_2O_2 in the presence of tempol. As a result, there is a smaller decrease in the intracellular concentration of hydrogen peroxide during hypoxia. Considering the theory that HPV is evoked by a sudden fall in H_2O_2 concentration, this could be one explanation of tempol-induced inhibition of HPV. The alternative explanation, arising from the possibility that the intracellular increase of ROS is an attribute of HPV, seems to be more likely, however. In tempol-induced potentiation of superoxide dismutation, the excess of H_2O_2 may be readily metabolized by catalase, and inhibition of HPV results from the deficient increase in ROS concentration. The intermediate for hypoxic pulmonary vasoconstriction could also be the superoxide molecule. Owing to its reactivity, however, it seems more likely that superoxide is rapidly converted to diffusible and significantly more stable hydrogen peroxide within the mitochondria (Burke & Wolin, 1987; Moudgil *et al.* 2005). Another potential mediator in HPV could be the hydroxyl radical produced in the Fenton reaction. Increased perfusion pressure was found in the presence of the hydroxyl radical, and this effect was subsequently inhibited by dimethylthiourea (an hydroxyl radical scavenger; Tate *et al.* 1982). However, the particular effect of the hydroxyl radical in hypoxic pulmonary vasoconstriction remains unclear.

Angiotensin II used in our experimental protocol served primarily for prestimulation of pulmonary vessels. We observed a significant decrease in ATII-induced vasoconstriction after administration of tempol. This finding may reflect an essential role of either superoxide or hydrogen peroxide in the vasoconstrictive response

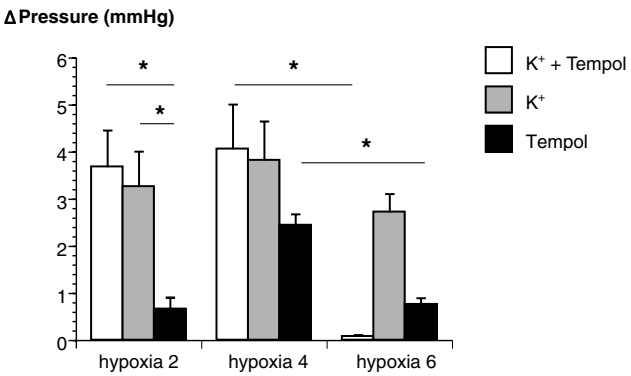

Figure 6. Effect of K^+ concentration on tempol-mediated inhibition of hypoxic pulmonary vasoconstriction
The bar graph shows the increase in perfusion pressure induced by acute ventilatory hypoxia (ΔP); $^*P < 0.05$.

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to ATII. Via activation of NADPH oxidase, ATII stimulates superoxide production in endothelial cells (de Gasparo, 2002; Lassegue & Clempus, 2003). Scavenging of superoxide by tempol then inhibits ATII-mediated vasoconstriction. In chronic experiments, the effects of ATII on renal vasculature are inhibited by tempol (Welch *et al.* 2005). It is not certain, however, that a similar mechanism can be ascribed to the fast vasoconstriction induced by bolus injection of ATII in lung vessels. Grimminger and co-workers blocked, in isolated rabbit lungs, the hypoxic pulmonary vasoconstriction induced by the NADPH oxidase inhibitor diphenyleneiodonium, whereas the vasoconstrictor response to ATII was not altered (Grimminger *et al.* 1995).

Aside from SOD-mediated reduction of superoxide into H_2O_2, superoxide reacts rapidly with NO, creating peroxynitrite. Although the exact role of NO production during HPV is not completely understood, many authors have observed that NO synthase inhibitors, such as N^G-monomethyl-L-arginine or L-NAME, significantly potentiate the vasoconstrictor responses of isolated rat lungs to hypoxia (Archer *et al.* 1989*b*; Barer *et al.* 1993; Hampl & Herget, 2000). Scavenging the superoxide using SOD or tempol prevents the peroxynitrite formation from NO, which consequently contributes to a higher concentration of available NO. Beckman *et al.* (1990) proposed that at least a part of the SOD protective effect may result from a decrease in the decomposition of NO. Lilley & Gibson (1996) also found that the role of antioxidants, such as superoxide dismutase, ascorbate or reduced glutathione, is in the protection of NO against superoxide anions. Since we found that tempol abolished vasoconstrictor responses in the same manner with or without pretreatment with L-NAME, its inhibition of HPV does not appear to result from a higher concentration of NO.

In the pathway of HPV, K_V channels are described as likely effectors (Reeve *et al.* 2001). Some of the K_V channels are sensitive to changes in redox potential. A decrease of superoxide radical or H_2O_2 during hypoxia causes conformational changes in these channels by reduction or oxidation of cysteine and methionine groups (Rettig *et al.* 1994). The resulting inhibition of K^+ current causes membrane depolarization, leading to the activation of Ca^{2+} entry via L-type Ca^{2+} channels, with subsequent myocyte contraction (Moudgil *et al.* 2005). The 'high ROS in HPV' alternative is that an increase in intracellular Ca^{2+} is the stimulus (Waypa *et al.* 2001). In isolated rat lungs, increasing the concentration of K^+ ions in the perfusate depolarizes the vascular smooth muscle and thereby increases the baseline vascular tone and potentiates the vasoconstrictor response to acute ventilatory hypoxia. When increasing K^+ concentration in the perfusate, we were interested in whether this could prevent the tempol-induced inhibition of HPV. Although K^+ administration significantly increased HPV, tempol almost completely abolished this vasoconstrictor response. In the presence of tempol, the hypoxic response was not dependent on the K^+ concentration in the perfusate. This conforms to the idea that the changes in ROS concentration are crucial in the onset of HPV.

In conclusion, the present study demonstrates that reduction of superoxide concentration by tempol inhibits HPV, and that the HPV inhibitory effect does not result from protection of NO against superoxide anions.

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