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Superoxide constricts rat pulmonary arteries via Rho-kinase-mediated Ca^{2+} sensitization

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Abstract

Reactive oxygen species play a key role in vascular disease, pulmonary hypertension, and hypoxic pulmonary vasoconstriction. We investigated contractile responses, intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), Rho-kinase translocation, and phosphorylation of the regulatory subunit of myosin phosphatase (MYPT-1) and of myosin light chain (MLC₂₀) in response to LY83583, a generator of superoxide anion, in small intrapulmonary arteries (IPA) of rat. LY83583 caused concentration-dependent constrictions in IPA and greatly enhanced submaximal $\text{PGF}_{2\alpha}$ -mediated precontraction. In small femoral or mesenteric arteries of rat, LY83583 alone was without effect, but it relaxed a $\text{PGF}_{2\alpha}$ -mediated precontraction. Constrictions in IPA were inhibited by superoxide dismutase and tempol, but not catalase, and were endothelium and guanylate cyclase independent. Constrictions were also inhibited by the Rho-kinase inhibitor Y27632 and the Src-family kinase inhibitor SU6656. LY83583 did not raise $[\text{Ca}^{2+}]_i$, but caused a Y27632-sensitive constriction in α -toxin-permeabilized IPA. LY83583 triggered translocation of Rho-kinase from the nucleus to the cytosol in pulmonary artery smooth muscle cells and enhanced phosphorylation of MYPT-1 at Thr-855 and of MLC₂₀ at Ser-19 in IPA. This enhancement was inhibited by superoxide dismutase and abolished by Y27632. Hydrogen peroxide did not activate Rho-kinase. We conclude that in rat small pulmonary artery, superoxide triggers Rho-kinase-mediated Ca^{2+} sensitization and vasoconstriction independent of hydrogen peroxide.

Keywords

Rho-kinase; Hypoxia; Superoxide; Hydrogen peroxide; Pulmonary; Free radicals

Reactive oxygen species (ROS), which include the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are important regulators of cell function in the vasculature and, when produced in excess (oxidative stress), contribute to endothelial dysfunction and proliferative diseases such as atherosclerosis, pulmonary hypertension, and right ventricular hypertrophy [1–4]. Superoxide is normally generated within the cell in response to agonists by

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oxidoreductase enzymes such as NADPH oxidase and xanthine oxidase [5–8] and in response to hypoxia by the mitochondrial respiratory chain [9,10]. Excessive superoxide can result from a deficiency in the expression or activity of the various antioxidant systems such as superoxide dismutase (SOD) and glutathione peroxidase and catalase [1]. This excess may cause uncoupling of endothelial nitric oxide synthase, inhibition of nitric oxide production, and production of yet more superoxide, along with other defects [1,3,11]. Both superoxide and H_2O_2 are modulators of vascular tone. Superoxide is usually procontractile in the pulmonary and other vascular beds [12–17], although it is not always clear whether it is acting directly or via its conversion to H_2O_2 [15]. H_2O_2 on the other hand has both proconstrictor and prorelaxant actions, depending on the type of vascular bed and experimental conditions used [16–21], but in our hands it constricts rat intrapulmonary artery [22].

The mechanisms underlying hypoxic pulmonary vasoconstriction (HPV), an adaptive process that optimizes pulmonary ventilation–perfusion matching in response to localized alveolar hypoxia, are not fully understood, but they involve changes in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) [10,23–25]; activation of protein kinases [26], particularly Rho-kinase [27,28]; and subsequent Ca^{2+} sensitization [29–31] and require an intact endothelium for a maximum and sustained response [32–34]. Several models have been proposed to explain how the pulmonary artery senses a reduction in oxygen tension. One of these suggests that hypoxia enhances production of superoxide from complex III of the mitochondrial electron transport chain [9,10]. It is generally assumed that superoxide is rapidly converted to H_2O_2 by mitochondrial Mn-SOD and cytosolic CuZn-SOD and that H_2O_2 , and not superoxide, acts as the signaling moiety that initiates the resulting vasoconstriction [10]. Indeed, strong evidence has been presented that H_2O_2 is responsible for the elevation of $[\text{Ca}^{2+}]_i$ in pulmonary artery smooth muscle cells during hypoxia [10,35]. Nevertheless, HPV has also been reported to be inhibited by SOD and enhanced by SOD antagonists [36,37], whereas, if H_2O_2 were the sole signaling moiety, one might expect SOD to be stimulatory and SOD antagonists inhibitory. However, the potential involvement of ROS in Rho-kinase-mediated Ca^{2+} sensitization in pulmonary artery remains to be investigated. It has been shown that exogenous superoxide generated by a xanthine/xanthine oxidase system activates the RhoA/Rho-kinase pathway in aorta [14], and cold-induced vasoconstriction of skin arteries is initiated by an increased production of mitochondrial ROS and subsequent activation of Rho-kinase [12].

In this study, we used LY83583, which generates intracellular superoxide by acting as a substrate for endogenous oxidoreductase enzymes [38], to investigate the mechanisms through which superoxide constricts small intrapulmonary arteries of rat. Our results have implications for the role of ROS in HPV and the wider field of vascular disease, particularly pulmonary hypertension, which is heavily influenced by Rho-kinase [39–41].

Materials and methods

Reagents and chemicals

Fura PE-3/AM was from Sigma (Poole, UK), dihydroethidium (DHE) and MitoSOX red were from Invitrogen (Paisley, UK), and the luminol derivative L-012 (8-amino-5-chloro-7-

phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione) was from Wako Chemicals USA (USA). Hydrogen peroxide, antioxidants, LY83583, and other pharmacological agents were from Amersham (Buckinghamshire, UK), Biomol (Exeter, UK), Calbiochem (Merck Biosciences, Nottingham, UK), Fisher (Loughborough, UK), Invitrogen, Pierce (Cramlington, UK), or Sigma (Poole, UK). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Upstate (UK), or Cell Signalling (UK), as described previously [42].

Tissue isolation and cell culture

This study conformed with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996). Intrapulmonary (IPA; first- to third-order branches) and mesenteric or femoral arteries (second- or third-order) were obtained from male Wistar rats (200–250 g). Pulmonary artery smooth muscle cells (PASMC) were dispersed enzymatically and grown in DMEM with 10% FCS to passage 4 or 5. Cells were growth-arrested in serum-free medium for 24 h before experiments, as described previously [42].

Measurement of superoxide

PASMC were grown on coverslips or in 96-well plates, for measurement of superoxide production. Because it has been suggested that no single ROS indicator is completely reliable [43], we performed these experiments with three different superoxide indicators: DHE, MitoSOX red, and L-012. For DHE and MitoSOX red fluorescence measurement, PASMC were preloaded with the dyes in DMEM for 20 min (10 or 5 $\mu\text{mol/L}$ for DHE or MitoSOX red, respectively) and then washed in medium before treatment with LY83583. Reactions were terminated by adding paraformaldehyde fixative. Intensity of staining within cells was taken as a measure of superoxide production. For L-012 luminosity measurement, PASMC were bathed in Hepes-buffered physiological salt solution and incubated with L-012 (10 μM) in the absence or presence of LY83583 (0–30 μM). Luminescence was measured with a luminometer (Chameleon; Hidex, Turku, Finland) after 30 min incubation. Measurements of L-012 luminescence for each LY83583 concentration in the absence of cells were subtracted as background.

Measurement of force, intracellular Ca^{2+} , and α -toxin permeabilization

Isometric tension was measured using a wire myograph with IPA bathed in physiological salt solution (PSS), at 37°C, pH 7.4, as described previously [32,34,42]. When required, endothelium was removed by rubbing the artery lumen with a human hair. $[\text{Ca}^{2+}]_i$ was measured in Fura PE-3-loaded, myograph-mounted IPA. Tension was recorded simultaneously with light emitted by the whole artery at >510 nm at excitation wavelengths of 340 and 380 nm. The ratio of the emission intensities ($R_{340/380}$) was taken as a measure of $[\text{Ca}^{2+}]_i$ as described previously [24,44]. For Ca^{2+} -sensitization experiments, myograph-mounted IPA were permeabilized with α -toxin and Ca^{2+} clamped with 10 mmol/L EGTA, as described previously [42]. For the benefit of statistical analysis, the effects of inhibitors were evaluated by applying LY83583 twice to each artery: a 10-min control constriction followed by a second 10-min constriction after preincubation with inhibitor.

Rho-kinase translocation

PASMC were treated for 10 min in DMEM at 37°C. Fixed cells were permeabilized with Triton X-100 and stained overnight with anti-ROCK-2 primary antibody (1:100; Santa Cruz Biotechnology) at 4°C followed by incubation with Alexa Fluor 488-labeled secondary antibody (1:300; Santa Cruz) for 2 h at room temperature. ROCK-2 translocation was expressed as the ratio of cytosol/nucleus intensities, as described previously [42]. Note that ROCK-2 is the isoform of Rho-kinase most associated with phosphorylation of MYPT-1 and Ca²⁺ sensitization in smooth muscle [45].

Western blot

IPA were prepared, treated, and homogenized and protein was extracted for Western blot as described previously [42]. Membranes were blocked for 1 h with 5% skimmed milk and probed with primary antibody overnight at 4°C (1:1000–1:5000 in 1% milk), followed by horseradish peroxidase-conjugated anti-IgG secondary antibody for 1 h at room temperature (1:5000 in 1% milk). Membranes were first probed with anti-phospho-antibodies, then stripped for 1 h (Pierce stripping buffer), reblocked, and reprobed with corresponding anti-total antibodies.

Statistical analysis

Data were analyzed with SigmaStat (Systat Software, San Jose, CA, USA). Comparisons of the effects of treatments against control and of inhibitors against treatments were performed by one-way ANOVA with appropriate post hoc tests. Pair-wise comparisons were done by Student *t* test. A probability of $p < 0.05$ was deemed significant.

Results

LY83583 generates superoxide in PASMC

LY83583 is known to generate intracellular superoxide by acting as a substrate for oxidoreductase enzymes within the cell, but may also generate superoxide outside the cell after intracellular modification [38]. In PASMC preloaded with the superoxide selective dye DHE (Figs. 1A–1D) or MitoSOX red (not shown), 10 µmol/L LY83583 caused enhancement of fluorescence, which was abolished by SOD, whereas SOD alone had no effect. LY83583-dependent superoxide generation in PASMC was also demonstrated with the extracellularly applied luminescent indicator L-012. Luminescence increased with increasing concentrations of LY83583, reaching a maximum at ~ 10 µmol/L with an apparent EC₅₀ of 1.4 ± 0.2 µmol/L (Fig. 1E). These results confirm that LY83583 generates superoxide in PASMC and that this effect can be prevented by SOD. Consistent with the selectivity of DHE and MitoSOX red for superoxide, 30 µM H₂O₂ had no effect on fluorescence (not shown).

LY83583 causes constriction in IPA

Constrictor responses to LY83583 were examined in IPA of rat. Ten minutes of exposures to 10 µmol/L LY83583 induced sustained, reversible, and reproducible contractions (Fig. 2A). Reproducibility was confirmed in 20 IPA, in which there was no significant change in

contraction amplitude between the first and the second 10-min exposures to LY83583 (first, $17 \pm 3\%$ of maximal constriction with 80 mM K^+ -substituted PSS (KPSS) vs second, $15 \pm 2\%$ KPSS). LY83583-induced constrictions were concentration-dependent, comparable to superoxide generation, reaching a maximum at $\sim 10 \mu\text{mol/L}$ with an EC_{50} of $1.7 \pm 0.2 \mu\text{mol/L}$ (Fig. 2B).

In the presence of a small degree of precontraction with $5 \mu\text{mol/L}$ $\text{PGF}_{2\alpha}$ (10–15% of KPSS), LY83583, at concentrations of 0.1 and $1 \mu\text{mol/L}$, which alone caused only very small contractions in IPA (~ 1 and 5% of KPSS, respectively), induced much larger responses (at $1 \mu\text{mol/L}$, these had mean amplitudes $>50\%$ of those evoked by KPSS, an apparent EC_{50} of between 0.1 and $1 \mu\text{mol/L}$). These responses were slightly increased by raising the concentration of LY83583 to $10 \mu\text{mol/L}$ (Figs. 2C and 2D). Similar enhancements were observed when IPA were precontracted with PSS containing 30 mmol/L K^+ (30 mmol/L K^+ , $7 \pm 2\%$ of KPSS vs 30 mmol/L K^+ + $1 \mu\text{M}$ LY83583, $39 \pm 10\%$ of KPSS, $n = 3$).

LY83583-mediated constriction in IPA is superoxide-dependent

Constrictions induced by $10 \mu\text{mol/L}$ LY83583 were almost abolished by the superoxide scavenger SOD (which converts superoxide into H_2O_2) and the membrane-permeant superoxide scavenger tempol (which removes superoxide by recombination), but were unaffected by catalase (which destroys H_2O_2) (Figs. 3A and 3B). SOD also nearly abolished the $1 \mu\text{mol/L}$ LY83583-mediated enhancement of the constriction to $5 \mu\text{mol/L}$ $\text{PGF}_{2\alpha}$, whereas catalase was without significant effect (Figs. 3C and 3D). SOD also inhibited the LY83583-mediated enhancement of a 30 mmol/L K^+ -induced precontraction (LY83583 + $\text{PGF}_{2\alpha}$: $79 \pm 5\%$ block, $p < 0.01$, $n = 6$; LY83583 + 30 mmol/L K^+ : $61 \pm 8\%$ block, $p < 0.05$, $n = 3$). LY83583-induced contraction is therefore mediated by superoxide and not via dismutation of superoxide into H_2O_2 by endogenous SOD.

Contractile responses were also obtained in IPA when superoxide was instead generated with menadione (maximum response at $\sim 10 \mu\text{mol/L}$) or with the combination of hypoxanthine (10–40 $\mu\text{mol/L}$) and xanthine oxidase (100–400 mU/ml), and the xanthine/xanthine oxidase-mediated constriction was also abolished by tempol (Supplementary Figs. S1 and S2).

LY83583-mediated constriction in IPA is not via inhibition of guanylate cyclase

As well as being a generator of superoxide, LY83583 is well known as an inhibitor of soluble guanylate cyclase [46]. We therefore tested whether the constrictor actions of LY83583 in IPA were via this pathway. A high concentration of the structurally unrelated guanylate cyclase inhibitor ODQ (10 μM ; $\text{EC}_{50} \sim 20 \text{ nM}$ [47]) did not itself cause any constriction of IPA and did not significantly alter the response to $10 \mu\text{M}$ LY83583 (Figs. 4A and 4B). On the other hand, like LY83583, ODQ did enhance $\text{PGF}_{2\alpha}$ -mediated constriction ($0.5 \mu\text{M}$ $\text{PGF}_{2\alpha}$), but in the presence of ODQ $1 \mu\text{M}$ LY83583 still caused further enhancement of this constriction (Figs. 4C and 4D). LY83583-mediated inhibition of guanylate cyclase does not therefore contribute significantly to the constriction caused by this drug in IPA.

We also eliminated the possibility that LY83583 was causing constriction via inhibition of endothelial NO production [3,13] or inhibition of glutathione reductase [48] or by an endogenous constrictor prostanoid produced as a result of cyclooxygenase stimulation [16]. As shown in Supplementary Fig. S3, neither endothelial removal, inhibition of e-NOS with L-NAME, nor inhibition of prostanoid TP receptors with SQ-29548 had any significant effect on LY83583-mediated constriction. The glutathione reductase inhibitor BCNU neither mimicked nor inhibited the effects of LY83583, but did enhance the LY83583-mediated constriction (Supplementary Fig. S3).

LY83583-mediated constriction in IPA is dependent on Rho-kinase and Src-family kinase and independent of changes in $[Ca^{2+}]_i$

Constrictions induced by 10 $\mu\text{mol/L}$ LY83583 were abolished by the Rho-kinase inhibitor Y27632 (3 $\mu\text{mol/L}$) and partially blocked by SU6656 (30 $\mu\text{mol/L}$), an inhibitor of Src-family kinases (Figs. 5A and 5B). In confirmation of the role of superoxide in the above responses, vasoconstriction induced by hypoxanthine/xanthine oxidase (10 $\mu\text{mol/L}$ and 100 mU/ml) was also abolished by Y27632 (Supplementary Fig. S2).

The effectiveness of Y27632 against LY83583-induced constriction led us to explore the possibility that superoxide was acting upon a Rho-kinase-mediated Ca^{2+} -sensitization pathway. Fig. 5C shows that in Fura PE-3-loaded IPA, 10 $\mu\text{mol/L}$ LY83583 did not cause any measurable increase in $[Ca^{2+}]_i$. Similar results were obtained in 10 other experiments. To corroborate this observation, we performed additional constriction experiments in α -toxin-permeabilized IPA, wherein $[Ca^{2+}]_i$ was clamped at pCa 6.9, and in the presence of cyclopiazonic acid to eliminate the involvement of Ca^{2+} release from intracellular stores. Under these conditions, LY83583 caused constriction and this was strongly inhibited by Y27632 (Fig. 5D).

LY83583 activates Rho-kinase and induces superoxide-dependent and Rho-kinase-mediated phosphorylation of MYPT-1 and MLC_{20} in IPA

To investigate further the role of Rho-kinase in the LY83583-mediated constriction we examined the effects of LY83583 on the translocation of ROCK-2 (Rho-kinase), an indicator of Rho-kinase activation, and on the phosphorylation of MYPT-1 and MLC_{20} , the principle pathway through which Rho-kinase mediates Ca^{2+} sensitization [29–31]. LY83583 (10 $\mu\text{mol/L}$) caused the translocation of ROCK-2 from nuclei to cytoplasm in PASM. H_2O_2 on the other hand did not induce ROCK-2 translocation (Fig. 6). The effect of LY83583 was comparable in magnitude to that observed with a high concentration of $PGF_{2\alpha}$ (not shown, but which we have described previously [42]).

In IPA, the phosphorylation of MYPT-1 at Thr-855 (equivalent to Thr-850 in humans), a target of Rho-kinase [31], and of MLC_{20} at Ser-19, the principle regulator of cross-bridge cycling, was enhanced by 10 $\mu\text{mol/L}$ LY83583. This enhancement was significantly blocked by SOD and abolished by Y27632. Basal phosphorylation of MYPT-1 was almost abolished by Y27632, as shown previously [42], but unaffected by SOD (Figs. 7A and 7C). Basal phosphorylation of MLC_{20} , however, was only partially reduced by Y27632 and not significantly affected by SOD (Figs. 7B and 7D).

LY83583 also significantly enhanced MYPT-1 and MLC₂₀ phosphorylation at 1 $\mu\text{mol/L}$, albeit to a lesser extent than 10 $\mu\text{mol/L}$, and when combined with 5 $\mu\text{mol/L}$ PGF_{2 α} , produced an enhancement of MYPT-1 phosphorylation that was similar to the sum of the two separate stimuli (LY83583 alone, $26 \pm 14\%$ increase, $n = 6$; PGF_{2 α} alone, $29 \pm 7\%$ increase, $n = 13$; LY83583 + PGF_{2 α} , $66 \pm 16\%$ increase, $n = 7$). On the other hand, the effect of the combined actions of 1 $\mu\text{mol/L}$ LY82583 and 5 $\mu\text{mol/L}$ PGF_{2 α} on MLC₂₀ phosphorylation was equivalent to almost twice that of the combined actions of 1 $\mu\text{mol/L}$ LY83583 and 5 $\mu\text{mol/L}$ PGF_{2 α} alone (LY83583 alone, $30 \pm 13\%$ increase, $n = 6$; PGF_{2 α} alone, $25 \pm 8\%$ increase, $n = 13$; LY83583 + PGF_{2 α} , $104 \pm 18\%$ increase, $n = 7$). This observation corroborates the apparent synergistic effect of 1 $\mu\text{mol/L}$ LY83583 and 5 $\mu\text{mol/L}$ PGF_{2 α} on contraction (as shown in Figs. 2C and 2D).

LY83583-mediated constriction and relaxation in systemic arteries

In contrast to IPA, mesenteric arteries were completely unresponsive to 10 $\mu\text{mol/L}$ LY83583, whereas femoral arteries produced constrictions approximately one-tenth the relative size of those in IPA (Figs. 8A and 8B). In the presence of a small 5 $\mu\text{mol/L}$ PGF_{2 α} -induced precontraction, 1 $\mu\text{mol/L}$ LY83583 did cause significant constrictions in both femoral and mesenteric arteries but these were still relatively small compared to IPA and generally transient. In addition, and in marked contrast to IPA, raising the LY83583 concentration to 10 $\mu\text{mol/L}$ relaxed both types of artery, such that the tension fell to a level significantly below that of the underlying PGF_{2 α} constriction (Figs. 8C and 8D). To determine whether these differences between IPA and systemic arteries were due to differential metabolism of superoxide and possible relaxing effects of H₂O₂ generated by endogenous SOD [49], we repeated these experiments in the presence of catalase. Catalase slightly enhanced constriction to 10 $\mu\text{mol/L}$ LY83583 in femoral artery and allowed a very small but significant constriction in mesenteric artery (Fig. 8B) and also significantly enhanced the response to 1 $\mu\text{mol/L}$ LY83583 in the presence of PGF_{2 α} in both artery types (Fig. 8D). However, catalase did not alter the relaxation caused by raising the concentration of LY83583 to 10 $\mu\text{mol/L}$ (Fig. 8D).

Discussion

The results of this study clearly show that the superoxide generating molecule LY83583 (at $> 1 \mu\text{mol/L}$), causes a sustained, reproducible, SOD- and Y27632-inhibitable constriction in small pulmonary arteries that does not require a change in $[\text{Ca}^{2+}]_i$ and is independent of the guanylate cyclase inhibitory action of this drug [46]. Our results show that LY83583-derived superoxide activates Rho-kinase, which phosphorylates MYPT-1, the regulatory subunit of myosin phosphatase, resulting in enhanced phosphorylation of the 20-kDa subunit of myosin light chain. Thus, superoxide anion causes Ca^{2+} sensitization [29–31]. The fact that Y27632 almost abolished all MYPT-1 phosphorylation at Thr-855, both basal and stimulated, confirms the observation that Rho-kinase is solely responsible for phosphorylating this site [42,50] and is therefore being activated by superoxide. Basal phosphorylation of MLC₂₀ was also partially blocked by Y27632, implying that Rho-kinase may contribute to resting tone in IPA, as we have previously suggested [42]. The fact that SOD affected only the LY83583-mediated increase in MYPT-1 phosphorylation and not basal phosphorylation suggests that

basal endogenous superoxide levels are not sufficient to cause Ca^{2+} sensitization in this preparation.

It is of note that extracellularly applied (non-cell permeative) SOD was effective against LY83583-mediated superoxide production, constriction, and MYPT-1 phosphorylation. Because we are potentially measuring superoxide both intracellularly and extracellularly (using DHE or MitoSOX red and L-012, respectively), the immediate site of action of superoxide in this model is unclear. Furthermore, whether SOD is entering cells or it is dismuting superoxide solely at the cell surface is not known. However, regardless of the mechanism, our results clearly indicate that SOD is lowering the superoxide concentration in PASMC and inhibiting the actions of LY83583 in IPA. In addition, the cell-permeative SOD mimetic tempol also inhibited LY83583-mediated constrictor responses.

LY83583-mediated constriction in IPA was independent of inhibition of guanylate cyclase or glutathione reductase by LY83583 [46,48], either directly or via superoxide, because constriction to LY83583 persisted in the presence of the structurally unrelated inhibitors ODQ and BCNU, respectively, which did not themselves cause constriction. The LY83583-mediated constriction in IPA also did not depend on an intact endothelium and was therefore independent of NO production or endogenous prostanoid release, despite evidence suggesting that the harmful vasoconstrictor and proliferative effects of superoxide in the vasculature are due to NO scavenging and subsequent uncoupling of e-NOS by peroxynitrite [1,3,11]. An independence of the vasoconstrictor actions of superoxide from endothelial function has been observed in a model of pulmonary hypertension, using xanthine/xanthine oxidase as a source of exogenous superoxide [4].

Although we deliberately used a high concentration of LY83583 to characterize the pure superoxide-mediated constriction, there were also effects of lower concentrations of LY83583 when applied in combination with other stimuli. These responses were also blocked by SOD and independent of guanylate cyclase inhibition. When measuring constriction and MLC_{20} phosphorylation, we found an apparent synergy between 0.1 or 1 $\mu\text{mol/L}$ LY83583 (which alone did not greatly raise tone) and either $\text{PGF}_{2\alpha}$ (which both elevates $[\text{Ca}^{2+}]_i$ and activates Rho-kinase [42,51]) or submaximal KCl (which principally elevates $[\text{Ca}^{2+}]_i$). The amount of superoxide generated within the cell required to trigger measurable constriction therefore depends on the level of submaximal activation of other pathways, particularly Ca^{2+} influx, which synergizes with Rho-kinase at the level of MLC_{20} phosphorylation. Low-level superoxide generation may also contribute to agonist-mediated constriction, probably via activation of oxidoreductase enzymes such as NADPH oxidase [5–8]. Indeed, in this study, $\text{PGF}_{2\alpha}$ -mediated constriction was partially sensitive to SOD. Synergy between superoxide generation and other contractile pathways may also be of relevance to HPV, because this is also dependent on Rho-kinase [27,28] and is strongly potentiated by a degree of precontraction with an agonist such as $\text{PGF}_{2\alpha}$ [32].

Our observation that the LY83583-mediated constriction was also partially blocked by inhibitors of Src-family kinases (SU6656) is also of interest. We have recently shown that $\text{PGF}_{2\alpha}$ -mediated constriction of rat IPA is dependent on activation of both Src-family kinases and Rho-kinase and that Src-family kinases are likely to be acting upstream of Rho-

kinase activation [42]. We have also found that Src-family kinases are activated by hypoxia [52]. Src-family kinases may act as intermediaries between ROS and Rho-kinase, perhaps by phosphorylating guanine-exchange factors or guanine-dissociation inhibitors, thus affecting RhoA translocation and therefore Rho-kinase activation [53,54]. This may tie in with the recent paper by Jernigan et al. [55], who demonstrated enhanced activity of RhoA as well as enhanced MYPT-1 phosphorylation in IPA of chronically hypoxic rats, which was apparently driven by superoxide [55].

There are several models for how ROS are affected by hypoxia and how they may contribute to HPV and responses to chronic hypoxia. The “redox hypothesis” proposes that ROS production is depressed by hypoxia and that the reduced cytosolic redox state inhibits voltage-gated K^+ channels, thus causing depolarization and Ca^{2+} influx [56]. In direct contrast, the “ROS hypothesis” maintains that mitochondrial ROS production is enhanced by hypoxia, leading to release of Ca^{2+} from intracellular stores and subsequent capacitative Ca^{2+} entry [9,10,57]. In the latter model, originally proposed by Waypa and Schumacker and colleagues [10], it is generally assumed that the constrictor response to hypoxia in pulmonary artery is entirely due to H_2O_2 , partly because of the short half-life of superoxide and the high concentration of mitochondrial SOD, but also because several studies have shown that overexpression of catalase or glutathione peroxidase suppresses the hypoxic-mediated elevation of $[Ca^{2+}]_i$ in PASMCM [10,35]. Our findings that both superoxide and H_2O_2 [22] cause sustained vasoconstriction of IPA are consistent with the concept that an elevation of ROS initiates HPV, but not with the redox hypothesis. However, the facts that LY83583-mediated constriction and MYPT-1 phosphorylation were blocked by SOD and tempol but not by catalase show that the observed effects were direct actions of superoxide and not a result of H_2O_2 produced by endogenous SOD. In support of this, we also showed that exogenous H_2O_2 did not activate Rho-kinase. This is also consistent with our recent observations that H_2O_2 caused Y27632-insensitive constriction and did not stimulate MYPT-1 phosphorylation [22]. Indeed it has been shown that HPV is enhanced by a SOD inhibitor and blocked by SOD itself [36,37]. On the other hand, H_2O_2 does indeed elevate $[Ca^{2+}]_i$ in IPA [22], whereas LY83583 apparently does not. We can speculate that mitochondrial superoxide drives Rho-kinase-mediated Ca^{2+} sensitization, whereas its product H_2O_2 drives the elevation in $[Ca^{2+}]_i$. Interestingly, H_2O_2 also does not enhance MLC₂₀ phosphorylation [22], suggesting a non-myosin-based mechanism of contraction, perhaps via actin polymerization or non-myosin-based regulation of cross-bridge cycling [58].

The pulmonary circulation is the only vascular bed in the adult mammal that constricts in response to hypoxia. In the systemic circulations, including mesenteric and femoral, hypoxia causes relaxation of precontracted arteries [32]. It is therefore of note that in femoral and mesenteric arteries, LY83583, at a concentration that caused constriction in IPA, either in the absence or in the presence of $PGF_{2\alpha}$, elicited no response in the absence of precontraction and a contraction at low dose followed by relaxation at high dose when applied in the presence of $PGF_{2\alpha}$. Bae et al. have shown that the superoxide generator menadione inhibits relaxation at low dose and inhibits contraction at high dose in rat aorta [49]. They proposed that at low dosage, superoxide causes constriction, but at high dose, there is H_2O_2 -dependent endothelium-independent relaxation. This is consistent with the idea of H_2O_2 being an

endothelium-derived hyperpolarizing factor [18–20]. Our results with LY83583 and catalase in mesenteric and femoral arteries may be partly explained by this model. Catalase did indeed enhance LY83583-mediated constriction, particularly at 1 $\mu\text{mol/L}$ LY83583 in the presence of $\text{PGF}_{2\alpha}$, but had no effect on these responses in IPA. However, catalase was ineffective against the relaxation induced by the higher concentration of LY83583. Whether this is due to poor penetration of catalase or an alternative superoxide-dependent relaxation pathway in systemic arteries remains to be determined.

In conclusion, we have shown that superoxide activates Rho-kinase, resulting in Ca^{2+} sensitization and constriction of rat IPA. Our results have implications for the roles of superoxide and H_2O_2 in HPV and for the wider field of vascular disease, particularly the vasoconstriction and vascular remodeling in pulmonary hypertension, in which both superoxide and Rho-kinase are involved [2,41].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ROS	reactive oxygen species
SOD	superoxide dismutase
HPV	hypoxic pulmonary vasoconstriction
PASMC	pulmonary artery smooth muscle cells
IPA	intrapulmonary artery
PSS	physiological salt solution
KPSS	K^+ -substituted physiological salt solution
DHE	dihydroethidium

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Fig. 1. Measurement of superoxide generation by LY83583 in pulmonary artery smooth muscle cells.

(A–C) Representative images of DHE-loaded PASMC, showing fluorescence under (A) control conditions, (B) 10 min exposure to 10 μmol/L LY83583, and (C) LY83583 after 30 min preincubation with 200 U/ml SOD. (D) Average effect of treatments in three or four cell lines. ** $p < 0.001$ vs control, †† $p < 0.001$ vs LY83583 alone. SOD alone was without effect. Similar results were obtained in PASMC loaded with MitoSOX red ($n = 4–6$, not shown). (E) Superoxide measurement in PASMC with L-012 (10 μmol/L), showing concentration-dependent LY83583-mediated increases in luminescence ($n = 5$).

Fig. 2. Contractile responses to LY83583 in IPA.

(A) Example trace of contraction in IPA in response to repeated 10-min exposures to 10 μmol/L LY83583. (B) Concentration dose response to LY83583 in IPA ($n = 6$). (C and D) Cumulative effects of 0.1, 1.0, and 10 μmol/L LY83583 on IPA precontracted with 5 μmol/L PGF_{2α} (* $p < 0.05$, ** $p < 0.001$ vs PGF_{2α}, $n = 3-15$).

Fig. 3. Effects of antioxidants on LY83583-induced contraction in IPA.

(A) Example trace of the effect of 200 U/ml SOD on contraction induced by 10 µmol/L LY83583 and (B) average effects of SOD, tempol (3 mmol/L), and catalase (200 U/ml) on this contraction (** $p < 0.001$ vs control, $n = 5-7$). There was no significant difference between the effects of SOD and tempol. (C) Example trace of the effect of SOD on constriction induced by 1 µmol/L LY83583 in the presence of 5 µmol/L PGF_{2α}. (D) Average effects of SOD and catalase on this constriction (* $p < 0.05$, ** $p < 0.001$ vs control, $n = 6$).

Fig. 4. Effects of guanylate cyclase inhibitor ODQ on LY83583 and PGF_{2 α} -induced constriction in IPA.

(A) Example trace of the effect of ODQ (10 $\mu\text{mol/L}$) on constriction induced by 10 $\mu\text{mol/L}$ LY83583. (B) ODQ did not significantly affect the size of the LY83583-induced constriction under these conditions ($n = 6$). (C) Example trace of the effect of ODQ on constriction induced by 1 $\mu\text{mol/L}$ LY83583 in the presence of 0.5 $\mu\text{mol/L}$ PGF_{2 α} . (D) Although it enhanced the PGF_{2 α} -induced constriction, ODQ did not significantly affect the size of the constriction induced by 1 $\mu\text{mol/L}$ LY83583 under these conditions ($n = 6$).

Fig. 5. LY83583-mediated constriction in IPA is Rho-kinase- and Src-family-kinase-dependent and intracellular Ca^{2+} -independent.

(A) Example trace of the effect of 3 $\mu\text{mol/L}$ Y27632 on constriction induced by 10 $\mu\text{mol/L}$ LY83583. (B) Average effects of Y27632 and the Src-family kinase inhibitor SU6656 (30 $\mu\text{mol/L}$) on this constriction (** $p < 0.001$ vs control, * $p < 0.01$ vs control, $n = 4-7$). Y27632 was also significantly more effective than SU6656 ($\dagger p < 0.01$). (C) Example traces showing lack of change in $[\text{Ca}^{2+}]_i$ induced by 10 $\mu\text{mol/L}$ LY83583 in Fura PE-3-loaded IPA (typical of 10 others, as measured by the ratio of emission intensity at >510 nm from excitation wavelengths of 340 and 380 nm; upper trace) and simultaneous tension measurement (lower trace) Response to KPSS (maximum is off scale) is included to verify successful loading of tissue with Fura PE-3. (D) LY83583-induced constriction (10 $\mu\text{mol/L}$) with $[\text{Ca}^{2+}]_i$ clamped at pCa 6.9 after permeabilization with α -toxin and subsequent reversal with 3 $\mu\text{mol/L}$ Y27632. Average responses in six arteries (* $p < 0.01$ vs pCa 6.9; $\dagger\dagger p < 0.001$ vs LY83583 alone).

Fig. 6. Effect of LY83583 on ROCK-2 translocation.

(A–C) Representative images of PASMC stained for ROCK-2 under control conditions (A) and after 10 min treatment with 10 $\mu\text{mol/L}$ LY83583 (B) or 30 $\mu\text{mol/L}$ H_2O_2 (C). (D) Average degree of translocation expressed as a ratio of nuclear/cytosolic staining intensities in cell lines derived from three to eight rats (** $p < 0.01$ vs control).

Fig. 7. Effects of LY83583 on phosphorylation of MYPT-1 and MLC₂₀.

(A and B) Representative Western blots of the effects of 10 $\mu\text{mol/L}$ LY83583 in the presence or not of 200 U/ml SOD or 3 $\mu\text{mol/L}$ Y27632 and of SOD and Y27632 alone on (A) MYPT-1 phosphorylation at Thr-855 or (B) MLC₂₀ phosphorylation at Ser-19. (C and D) Average effects of LY83583 alone ($n = 13$), LY83583 plus SOD ($n = 11$), LY83583 plus Y27632 ($n = 7$), SOD alone ($n = 15$), and Y27632 alone ($n = 9$), expressed as a percentage of control (untreated) phosphorylation of (C) MYPT-1 and (D) MLC₂₀. * $p < 0.05$ vs control, ** $p < 0.01$ vs control, † $p < 0.05$ vs LY83583 alone, †† $p < 0.001$ vs LY83583 alone.

Fig. 8. Constrictor and relaxant effects of LY83583 in mesenteric and femoral arteries.

(A) Example trace showing the lack of effect of 10 μmol/L LY83583 in mesenteric artery.

(B) Average constrictor effect of 10 μmol/L LY83583 in mesenteric and femoral arteries in the absence and presence of catalase (200 U/ml; * $p < 0.05$, $n = 7$ or 8).

(C) Example trace of cumulative effects of 1 and 10 μmol/L LY83583 on mesenteric artery precontracted with 5 μmol/L $\text{PGF}_{2\alpha}$.

(D) Average constrictor effects of LY83583 in femoral and mesenteric arteries in the absence and presence of catalase († $p < 0.05$ for 10 μmol/L vs 1 μmol/L LY83583; * $p < 0.05$ for presence vs absence of catalase, $n = 9$ or 10). *Note:* The axis in (D) is scaled the same as in Fig. 2D, for comparison, but the axis in (B) is scaled down for the sake of clarity.