Oxygen Activates the Rho/Rho-Kinase Pathway and Induces RhoB and ROCK-1 Expression in Human and Rabbit Ductus Arteriosus by Increasing Mitochondria-Derived Reactive Oxygen Species

A Newly Recognized Mechanism for Sustaining Ductal Constriction

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Background—Constriction of the ductus arteriosus (DA) is initiated at birth by inhibition of O2-sensitive K+ channels in DA smooth muscle cells. Subsequent membrane depolarization and calcium influx through L-type calcium channels initiates functional closure. We hypothesize that Rho-kinase activation is an additional mechanism that sustains DA constriction.

Methods and Results—The effect of increased PO2 on the activity and expression of Rho-kinase was assessed in DAs from neonates with hypoplastic left-heart syndrome (n=15) and rabbits (339 term and 99 preterm rabbits). Rho-kinase inhibitors (Y-27632 and fasudil) prevent and reverse O2 constriction. Heterogeneity exists in the sensitivity of constrictors (PO2=endothelin=phenylephrine>KCl) and of fetal vessels (DA=pulmonary artery>aorta) to Rho-kinase inhibition. Inhibition of L-type calcium channels (nifedipine) or removal of extracellular calcium inhibits approximately two thirds of O2 constriction. Residual DA constriction reflects calcium sensitization, which persists after removal of extracellular calcium and blocking of sarcoplasmic reticulum Ca2+-ATPase. In term DA, an increase in PO2 activates Rho-kinase and thereby increases RhoB and ROCK-1 expression. Activation of Rho-kinase in DA smooth muscle cells is initiated by a PO2-dependent, rotenone-sensitive increase in mitochondrion-derived reactive O2 species. O2 effects on Rho-kinase are mimicked by exogenous H2O2. In preterm DAs, immaturity of mitochondrial reactive oxygen species generation is associated with reduced and delayed O2 constriction and lack of PO2-dependent upregulation of Rho-kinase expression.

Conclusions—O2 activates Rho-kinase and increases Rho-kinase expression in term DA smooth muscle cells by a redox-regulated, positive-feedback mechanism that promotes sustained vasoconstriction. Conversely, Rho-kinase inhibitors may be useful in maintaining DA patency, as a bridge to congenital heart surgery. (Circulation. 2007;115:1777-1788.)

Key Words: oxygen ■ calcium ■ cardiovascular diseases ■ ductus arteriosus, patent ■ muscle, smooth ■ congenital heart disease ■ prematurity

In utero, the ductus arteriosus (DA) shunts more than half of the blood entering the right heart away from the unventilated fetal lung into the umbilicalplacental circulation, where gas exchange occurs. At birth, O2-induced DA constriction initiates functional closure of the DA within minutes. This is followed by a slower process of anatomic closure, which in humans requires several days and involves proliferation and apoptosis of DA smooth muscle cells (DASMCs). Robust, reversible O2-induced DA constriction is readily demonstrated in isolated DA rings and, although modulated by the endothelium (reinforced by the constrictor endothelin and attenuated by vasodilatory prostaglandin), persists in endo-

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thelium-denuded rings. Thus, the core of O₂-induced constriction is intrinsic to the DASMCl, 4-7 In rabbit and human DA, O₂ constriction is initiated in part by inhibition of O₂-sensitive, voltage-gated potassium channels (eg, Kv1.5, Kv2.1). 9-11 K⁺ channel inhibition depolarizes the DASMCl, which activates the voltage-sensitive L-type calcium channel and initiates vasoconstriction that is dependent on calcium influx. 4 This ionic mechanism is initiated in DASMCl in minutes of the rise in P O₂ from fetal (≈40 mm Hg) to newborn (>80 mm Hg) levels by a mitochondrial redox sensor that increases production of reactive oxygen species (ROS), particularly H₂O₂, in proportion to P O₂. 12

Smooth muscle contraction is primarily regulated by phosphorylation/dephosphorylation of myosin light chain (MLC). 13 MLC is phosphorylated by Ca²⁺/calmodulin-dependent MLC kinase and dephosphorylated by the Ca²⁺-independent MLC phosphatase (MLCP). Increased cytosolic Ca²⁺ activates MLC kinase, which leads to MLC phosphorylation and vasoconstriction. However, activation of the Rho-kinase pathway can induce calcium sensitization, 13,14 a phenomenon in which sustained vasoconstriction occurs, independent of ongoing increases in cytosolic Ca²⁺, due to MLCP inhibition and the resulting persistence of MLC phosphorylation. 13 Many G-protein receptor agonists (eg, phenylephrine [PE] and endothelin) produce contraction both by increasing cytosolic Ca²⁺ (by calcium influx or release of calcium from the sarcoplasmic reticulum [SR]) and induction of Rho-kinase-mediated Ca²⁺ sensitization.

The actions of the Rho family of GTPases (Rho, Rac, and CDC42) are mediated by their specific downstream effector, Rho-associated coiled-coil forming protein kinase (ROCK), 14 a serine-threonine kinase. 13,15,16 Rho-kinase is specifically inhibited by Y-27632 17 or fasudil (HA1077). 18 Although activation of the Rho/Rho-kinase pathway is known to be involved in actin stabilization, cell migration, tumor invasiveness, and regulation of vascular tone, 13 its role in the human DA and in O₂ sensing is largely unknown. Two recent reports highlight a potential role for Rho-kinase in the DA, one showing that Rho-kinase inhibitors relax endothelium-denuded rabbit DA 19 and the other indicating that RhoB gene expression increases with maturation in rat DA. 20 Although the role of RhoB is unknown, the latter observation is intriguing because although DA closure rarely fails in term infants, persistent DA (patency beyond day 3) afflicts 21% of preterm infants. 21 We hypothesized that O₂ activates the Rho/Rho-kinase pathway, leading to sustained constriction, in term human and rabbit DA and that the mechanism of O₂-induced Rho-kinase activation in the DA involves a redox-dependent increase in both the activity and expression of components of the Rho-kinase pathway.

Methods

Isolated Human DA

Parents consented to discard tissue obtained during surgery used for research and confirmed this by signing the surgical consent form approved by the University of Alberta’s institutional review board. The DAs, which were all patent due to in vivo prostaglandin E1 infusion, were excised from neonates with hypoplastic left heart syndrome (n=15, mean age=10±1 days, 9 males) during the palliative Norwood procedure. DAs were immediately placed in iced saline in the operating room and within 20 minutes were suspended in Krebs’ solution, containing meclofenamate (10⁻⁵ mol/L, to inhibit prostaglandin synthesis) and N⁶-nitro-L-arginine methyl ester (10⁻⁴ mol/L, to inhibit nitric oxide synthesis). 4 DAs were studied at an experimentally determined, optimal passive tension of 1000 mg (defined by the maximal constriction to KCl 80 mmol/L). After 1 hour of equilibration in hypoxia (P O₂, 40 mm Hg), the DA was exposed to a normoxic solution (P O₂, 120 mm Hg). An O₂ electrode in the ring bath permitted simultaneous assessment of time and P O₂. The effect on tone of 2 chemically distinct Rho-kinase inhibitors (Y-27632 [10⁻⁵-10⁻³ mol/L] and fasudil [10⁻⁵-10⁻³ mol/L] 22) was assessed by administering them either at peak O₂ constriction or 30 minutes before P O₂ increased.

Isolated Rabbit DA

New Zealand White rabbits were delivered by caesarian section at term or preterm (30 and 26 days, respectively), and pups were euthanized with an overdose of pentobarbital (100 mg/kg IP). The activity and expression of the Rho-kinase pathway and its contribution to vasoconstriction was compared in DAs, third-branch pulmonary artery (PA), and descending thoracic aorta. Within 5 minutes of harvest, arteries were suspended in tissue baths and studied with the same solutions and normoxic/hypoxic P O₂, as in the human DA experiments. Optimal passive ring tensions were 800 mg (term DA), 400 mg (preterm DA), 600 mg (PA), and 1000 mg (aorta). In DA, the vasodilatory effect of Y-27632 (10⁻⁷ to 5×10⁻⁶ mol/L) or fasudil (10⁻⁸ to 5×10⁻⁷ mol/L) was assessed at peak O₂ constriction by application of incremental doses at 5-minute intervals. To assess possible agonist-specific vasodilator heterogeneity, the effects of Y-27632 and fasudil were compared in hypoxic arteries constricted with endothelin (10⁻⁷ mol/L), KCl (80 mmol/L), or PE (10⁻⁷ mol/L). To determine the relative contribution of net calcium influx versus calcium influx via the L-type voltage-gated calcium channel, O₂ constriction was assessed in the presence of nifedipine (10⁻⁶ mol/L) or 0 mmol/L Ca²⁺. To exclude a role for SR Ca²⁺ release, we also studied O₂- and PE-induced DA constriction in 0 mmol/L Ca²⁺ Krebs’ solution containing cyclopiazonic acid (10⁻³ mol/L), an SR Ca²⁺-ATPase inhibitor. Temporal changes in the importance of Rho-kinase to O₂-induced DA constriction was assessed by administering Y-27632 (10⁻⁷ to 5×10⁻⁷ mol/L) at varying durations of normoxic exposure (30 to 480 minutes).

Quantitative Reverse-Transcription Polymerase Chain Reaction, Immunoblotting, and Immunofluorescence

The effect of increasing P O₂ for 1 to 2 hours on the activity and expression of components of the Rho/ROCK pathway was assessed in freshly isolated human term DA, rabbit DAs (term and preterm), and human DASMCl (see online Data Supplement).

RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

Arteries were isolated and immediately frozen in liquid nitrogen. RNA was isolated with the RNeasy Plus Mini Kit (Qiagen: Mississauga, Ontario, Canada) and quantified with ultraviolet spectrophotometry. The following primers, synthesized by Applied Biosystems (Foster City, Calif), were used: RhoA (Rn00589172_m1), RhoB (Rn00579404_s1), and ROCK-1 (Hs00178463_m1, Rn00579490_m1). mRNA levels were measured with the TaqMan One-Step RT-PCR Master Mix reagent kit (Applied Biosystems) and expressed as 2ΔΔCt, which normalizes gene expression to a ribosomal 18S reporter and a calibrator sample, as described previously. 10

Immunoblotting

Arteries were flash-frozen in liquid nitrogen and homogenized in buffer containing an antiprotease cocktail before electrophoresis on 7.5% or 15% SDS-PAGE gels. Expression, relative to a reporter (α-actin unless otherwise stated), was quantified by densitometry. Sources for all reagents and antibodies (RhoA, RhoB, ROCK-1,
ROCK-2, ROCK1 cleavage site [1113/1114] antibody, phospho-MYPT [myosin phosphatase target] [Thr696], MYPT, phospho-CPI-17 Thr38, and CPI-17) are listed in the online Data Supplement.

**RhoA Activity**

RhoA activity was assessed in homogenized arteries samples by an immunoprecipitation assay, which targeted the Rho-binding domain of the Rho effector protein (Rhotekin), as described previously. RhoA activity was defined as the ratio of precipitated GTP-bound RhoA/total RhoA ratio on the immunoblot.

**Confocal Microscopy**

Immunofluorescence was performed on DA cells or paraffin-embedded, formaldehyde-fixed human and rabbit DA sections, according to an antigen-retrieval protocol, with a Zeiss 510, 2-photon, confocal microscope, as described previously. After 1-hour incubation with primary antibody (36°C), slides were incubated for 45 minutes with the secondary antibody. Nuclear staining was performed with 4',6-diamidino-2-phenylindole, and slides were imaged (excitation/emission; green: 488 nm/505 to 530 nm, red: 543 nm/615 nm, and blue: 740 nm/390 to 465 nm). Imaging conditions were kept constant between each experimental group, and appropriate controls were performed in all cases (including imaging without antibodies to exclude autofluorescence and use of the secondary antibody alone, to exclude nonspecific staining).

**Measurement of Mitochondrial Superoxide Radical Production and H$_2$O$_2$ Release**

Mitochondrial superoxide generation was measured in live DASMC cells during 1 hour of hypoxia (Po$_2$ ~40 mm Hg) or normoxic (Po$_2$ ~120 mm Hg) with MitoSOX Red, a mitochondrial superoxide indicator that increases red emission in proportion to superoxide production (Molecular Probes, Eugene, Ore). Cells were loaded with MitoSOX Red (5×10$^{-6}$ mol/L) and the nuclear stain Hoechst 33342 (10$^{-6}$ mol/L) for 10 minutes (37°C), as described previously. Rotenone (10$^{-3}$ mol/L) was used to assess the contribution of electron transport chain complex I to mitochondrial ROS production. H$_2$O$_2$ release from DASMCs was measured with the AmplexRed assay (Molecular Probes), as described previously and in the online Data Supplement.

**Statistics**

Values are reported as mean±SEM, and sample sizes are stated on the Figures. For the isometric contraction experiments, n refers to the number of animals from which tissue was obtained. Intergroup comparisons were performed with a 2-tail, unpaired $t$ test or a factorial, repeated-measures ANOVA, as appropriate. A value of $P<0.05$ was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written. The Animal Welfare and the Human Studies committees of the University of Alberta approved all procedures.

**Results**

Rho-Kinase Inhibition Prevents and Reverses O$_2$ Constriction in Human DA

O$_2$ constriction begins within 4.6±1.2 minutes of a rise in Po$_2$ in human DA. Fasudil and Y-27632 decreased established
PO2-dependent constriction in a dose-dependent fashion (Figure 1A) and were even effective in preventing O2 constriction (Figure 1B and 1D). In addition, both Rho-kinase inhibitors (Figure 1B and 1C), but not nifedipine (online Data Supplement Figure IA), decreased the passive hypoxic tension below baseline.

Heterogeneity of the Vasodilator Effects of Rho-Kinase Inhibition in Rabbit Arteries
Rho-kinase inhibitors also reversed O2 constriction in rabbit DA (Figure 2A), and the longer the O2 exposure, the greater the relaxation by Y-27632 (Figure 2B). Rho-kinase inhibition was equally effective in reversing DA constriction induced by endothelin, PE, and O2; however, relaxation was less in KCl-constricted DA (Figure 2C). Although Y-27632 and fasudil reduced endothelin contraction in all arteries tested, relaxation was greater in DA and PA than in aorta (Figure 2D).

Calcium Sensitization Contributes to O2 Constriction in Rabbit DA
Although removal of extracellular Ca2+ or addition of nifedipine reduced O2 constriction by approximately two thirds, a residual one third of the constriction persisted (Figure 3A and 3B). When Ca2+ stores were depleted by removing extracellular calcium and SR stores were simultaneously depleted with cyclopiazonic acid (10−3 mol/L), approximately one third of O2 constriction (data not shown) and PE constriction persisted (Figure 3B), consistent with the occurrence of Ca2+ sensitization in DA. Y-27632 completely reversed O2 constriction in rabbit DA whether in the presence (Figures 2A and 3C) or absence (Figure 3C) of extracellular Ca2+.

Expression of Components of the Rho/Rho-Kinase Pathway in Human DA
Basal expression of RhoA, RhoB, ROCK-1, and ROCK-2 occurs in the DASMC and vasa vasora of freshly isolated, hypoxic human DA rings (Figure 4A through 4C). One hour of incubation in 20% O2 ex vivo increases RhoA, RhoB, and ROCK-1 protein expression in human DASMCs (Figure 4B through 4D).

O2 Activates Rho-Kinase and Upregulates Expression of RhoB and ROCK-1 in Term Rabbit DA
In experiments in which fetal arteries were incubated in normoxic Krebs’ solution (PO2 124±2 versus 39±5 mm Hg)
for 1 hour, DA RhoB protein expression was increased (Figure 5A). When Po2 was increased by having pups breathe room air for 1 hour, O2 increased mRNA levels of RhoB in DA and ROCK-1 in DA, PA, and aortas (Figure 5B); however, ROCK-1 protein was only increased in the DA (Figure 5C). To differentiate the relative contribution of Rho-kinase versus protein kinase C–mediated inhibition of MLCP to O2-induce Rho-kinase activation, we measured phosphorylation of MYPT-1 (mediated by Rho-kinase) versus CPI-17-PP1c (mediated by protein kinase C).25 Exposure to O2 (20% for 15, 30, and 60 minutes) significantly increased MYPT-1 phosphorylation at its inhibitory site (Thr696; Figure 5D) without altering CPI-17 phosphorylation at its inhibitory site (Thr38; Data Supplement Figure IB). Together with the failure of O2 to activate RhoA (no increase in GTP-RhoA; Figure 5A), this indicates Rho-kinase activation in DA is independent of RhoA or protein kinase C.

O2-Dependent Mitochondrial ROS Generation Activates Rho-Kinase in Human DASMCs

Because O2-dependent Rho-kinase activation occurred in the absence of RhoA-GTP complex, we assessed the possibility that a direct, distal activation of the pathway had occurred. Because increases in Po2 have previously been shown to rapidly increase mitochondrion-derived H2O2 production in human DA (triggering the early ionic events),12 we tested the ability of exogenous H2O2 (10 \( \mu \text{mol/L} \)) to induce Rho-kinase expression and activity. H2O2 increased expression of RhoB and ROCK-1 (Figure 6A through 6C) and also activated ROCK-1 (evident from the increase in levels of the cleaved C-terminal fragment; Figure 6B). Increasing Po2 also increased the endogenous production of H2O2 and mitochondrial superoxide generation by DASMCs (Figure 6D and 6E).

**Immaturity of the Mitochondrial Redox Mechanism for Increasing Rho-Kinase Expression in Preterm Rabbit DA**

The basal hypoxic level of mitochondrial superoxide was higher in term than in preterm rabbit DASMCs (Figure 7A). In response to increased Po2, superoxide production increased faster and to higher levels in term than in preterm rabbit DASMCs. In both term and preterm DA, the superoxide source measured by MitoSOX was confirmed to be mito-
chondria, based on its complete inhibition by rotenone (10⁻⁵ mol/L). Term, but not preterm, DASMCs increased H₂O₂ in response to increased PO₂ (Figure 7B). Consistent with the immaturity of the mitochondrial redox sensor, basal levels of ROCK-1 were lower in preterm DA, and O₂-dependent increases in ROCK-1 and RhoB expressions were absent (Figure 7C and 7D). The physiological correlate of this immature mitochondrial redox sensing mechanism was a smaller and slower onset of O₂ constriction in preterm rabbit DA (online Data Supplement Figure IIA and IIB).

Discussion
The temporal sequence of O₂ constriction of human (and rabbit) DA involves an early electrical phase (Kv channel inhibition, membrane depolarization, and activation of the L-type, voltage-gated calcium channel), followed by a mediator phase (increased endothelin synthesis), and finally, as this work shows for the first time, a calcium-sensitization phase (activation of Rho-kinase; Figure 8). The electrical phase begins within 5 to 10 minutes of rising PO₂ (Figures 1A, 1B, and 2A and Data Supplement Figure IIA), and the constriction is largely dependent on extracellular calcium (Figure 3A and 3B). In contrast, the mediator phase, not assessed in the present study, begins after ~30 minutes and likely involves release of intracellular calcium, in response to agonists like endothelin. Finally, largely owing to activation of Rho-kinase, the calcium-sensitization phase occurs and maintains constriction while reducing the requirement for calcium influx (Figure 3C).

A particular strength of the current work is that much of the physiology and dissection of cellular mechanisms was accomplished in human DAs, all of which had normal constrictor responses to O₂. This tissue is very rare and was handled carefully and rapidly to preserve its O₂ response. The use of human tissue reduces the need for extrapolation and ensures that the newly discovered mechanisms of O₂-induced, ROS-mediated Rho-kinase activation and upregulation are relevant to human infants. The present study has 4 major new findings. First, we demonstrate that Rho-kinase activation is necessary for sustained O₂ constriction and that calcium sensitization accounts for approximately one third of O₂ constriction (Figures 1 through 3). Second, with more pro-
longed O₂ exposure, the Rho-kinase pathway increases its contribution to DA constriction (Figure 2B). Third, O₂-induced Rho-kinase activation increases the expression of key components of the pathway in the DASMCs and vasa vasora, notably RhoB and ROCK-1, without activating RhoA-GTP (Figures 4, 5, and 7). This constitutes a form of a positive-feedback loop (Rho-kinase activity inducing Rho-kinase expression; Figure 8) that is absent in preterm DA (Figure 7). Fourth, we show that both Rho-kinase activation and increased expression occur via a redox mechanism that involves an O₂-dependent increase in mitochondrial ROS production in human and rabbit DA (Figures 6 and 7). The finding that increased O₂ increases endogenous ROS/H₂O₂ production and that exogenous H₂O₂ can increase ROCK-1 expression and activity (measured as increased levels of a cleavage site–specific form of ROCK-1; Figure 6A and 6B) is consistent with prior work showing that increased H₂O₂ production initiates Kv channel inhibition and functional DA closure.¹²,²⁷

Calcium influx plays an important role in eliciting DA constriction. In rabbit DA, we previously reported that virtually all DA constriction could be eliminated by blocking calcium entry with lanthanum or an L-type calcium channel blocker.⁴ Likewise, in human DA, most of the constriction to the Kv channel blocker 4-aminopyridine is inhibited by the lipophilic calcium channel blocker, nifedipine.⁸ The present data show that 70% of O₂ constriction is inhibited by removing extracellular calcium or blocking the L-type calcium channels (Figure 3A and 3B). The remaining 30% of constriction appears to be due to calcium sensitization rather than SR calcium release, because it is resistant to cyclopiazonic acid (Figure 3B). Hong et al.¹⁹ found that 48±7% of total O₂ constriction persists after nifedipine (10⁻⁶ mol/L) treatment. This nifedipine-resistant constriction (which was dependent on calcium influx) was attributed to activation of store-operated channels (TRP channels).¹⁹ Although we did not examine the role of TRP channels, we found a greater role for the L-type calcium channel and a smaller percent of O₂ constriction persisting after nifedipine (35±9% in human DA). This difference may reflect the recognized species differences in DA constriction¹ or differences in our experi-

![Figure 5.](http://circ.ahajournals.org/)
mental protocol (we used meclofenamate and nitro-L-arginine methyl ester, whereas Hong et al mechanically denuded the endothelium).

The present study demonstrates that Rho-kinase activation is crucial both to determining basal tension and to sustaining constriction in response to oxygen in term human DA (Figure 1). Consistent with this, 2 chemically discrete Rho-kinase inhibitors (fasudil and Y-27632) caused a dose-dependent attenuation of established O2 constriction, and if given during hypoxia, these inhibitors decreased basal tension (Figure 1B and 1C).

As expected, Rho-kinase activation is a universal, distal step in both O2- and agonist-induced DA constrictions. Consistent with prior observations in PA and aorta,22,28–31 fetal DA, PA, and aorta all relaxed in response to Y-27632, a highly selective inhibitor of the downstream kinase effector, ROCK-1.31 Nonetheless, we did detect some heterogeneity in the importance of Rho-kinase depending on the artery and vasoconstrictor studied. Y-27632 and fasudil induced greater relaxation (of endothelin constriction) in DA and PA than in aortas (Figure 2D). If enhanced by rational drug design, this modest DA specificity could be exploited to minimize undesired systemic hypotension if a Rho-kinase inhibitor were to be used in vivo to maintain DA patency, as a bridge to palliative congenital heart surgery. In addition, Rho-kinase inhibition, although equally effective in reversing constriction to oxygen, endothelin, and PE was less effective in reducing KCl constriction (Figure 2C). This may reflect the relatively greater dependence of KCl constriction on membrane depolarization and calcium entry via the L-type calcium channel.

Several lines of evidence show that brief incubation in O2 activates Rho-kinase pathway, and increased PO2 elevates mitochondrial ROS production. A, Representative immunofluorescent staining and mean intensity data of hypoxic DASMCs incubated with vehicle (left) vs H2O2 (10^{-5} mol/L) for 2 (middle) vs 4 (right) hours. H2O2 induces expression of RhoB and ROCK-1 (green). Nuclei are stained blue with 4',6-diamidino-2-phenylindole. B, Representative immunoblot and corresponding densitometric analysis demonstrates that H2O2 (10^{-5} mol/L, for 4 hours of incubation in hypoxia) increases expression of RhoB and ROCK-1 expression and the active form of ROCK-1 (measured with a cleavage site–specific antibody). C, H2O2 increases ROCK-1 mRNA expression. D, Mean data of AmplexRed show that H2O2 production of DASMCs increases after 30 minutes of normoxic incubation. E, Representative immunofluorescent staining and mean MitoSOX fluorescent intensity per cell. O2 (1 hour) increases DASMC mitochondrial superoxide production.
mRNA and protein levels is not exact. Notably, increased O2 increased phosphorylation of Thr696 in MYPT-1 (Figure 5D). Phosphorylation at MYPT-125,31–33 is known to inhibit MLCP activity, thereby stimulating MLC phosphorylation and contraction.34 In addition, we compared RhoA activity in DA rings at birth versus 1-hour O2 incubation. We determined the amount of active RhoA with use of a GST-Rhotekin “pull-down assay” (Rhotekin is a target of Rho).23 After 1-hour O2, the ratio of GTP-RhoA to total RhoA (the activity) was not increased. This surprising finding further indicates that the Rho-kinase pathway in DA is activated by a different mechanism than classically occurs with agonists, such as endothelin (Figure 8). We next established a mechanism by which rapid upregulation of RhoB and ROCK-1 occurs, namely, through ROS-mediated activation of Rho-kinase.

The present data show that exogenous H2O2 mimics the effects achieved by increasing PO2, namely, increased activity and expression of the Rho-kinase pathway (Figure 6A through 6C). The ability of ROS to activate the Rho/ROCK pathway, leading to vasoconstriction, has recently been reported in systemic arteries of animals,35,36 where it has been suggested as a potential basis for cold-induced vasospasm (Raynaud’s phenomenon). To the best of our knowledge, this redox-mediated increase in the activation and expression of Rho-kinase has not been reported previously in human arteries, nor has its role in the constriction of the DA been suggested. Not only does exogenous H2O2 activate Rho-kinase (Figure 6B) and increase expression of ROCK-1 (Figure 6A and 6B), but the rise in PO2 also elicits increased endogenous production of ROS by the mitochondria (Figure 6D and 6E). The fact that rotenone, an inhibitor of complex I that is known to mimic hypoxia and cause relaxation of the human DA,12 obliterates the O2-induced ROS production (Figure 7A) supports the central role of the mitochondria as vascular O2 sensors, controlling both the ionic and the calcium-sensitization phase of DA constriction (Figure 8).

The only aspect of the present work that was not directly confirmed in human DA is the finding that immaturity of mitochondrial ROS generation is associated with reduced and delayed onset of O2 constriction (Data Supplement Figure 8).
IIA) and lack of \( \text{PO}_2 \)-dependent upregulation of Rho-kinase expression in preterm rabbits (Figure 7C and 7D). This relates to our inability to obtain fresh, preterm human DAs. This leads us to speculate that immaturity of this pathway may contribute to the high prevalence of persistent DA in preterm animals (although other factors, such as decreased expression of \( \text{O}_2 \)-sensitive Kv channels, are also involved).\(^\text{10}\) Although Rho-kinase induction does not occur in preterm DAs, the Rho-kinase activity remains important, as evident by the complete relaxation of preterm DAs to Y-27632 (online Data Supplement Figure IIB).

Doses of Y-27632 or fasudil that inhibit \( \text{O}_2 \) constriction (Figures 1 and 2) also prevent an \( \text{O}_2 \)-induced increase in expression of pathway components (Figure 4C). This indicates that activation of the Rho-kinase pathway is participating in its own transcriptional regulation, which constitutes a form of positive feedback.\(^\text{37}\) This combined increase in enzyme activity and expression is likely a fail-safe mechanism and may explain the extremely low incidence of persistent DA in term infants. The fact that induction of the Rho-kinase pathway is unique to the DA (we noted no increase in ROCK-1 or RhoB protein in PA or aorta; Figure 5C) is likely beneficial, because the fetal PA must dilate, whereas the adjacent DA constricts, in response to rising \( \text{PO}_2 \).

The finding that upregulation of RhoB mRNA in arteries 1 hour after birth is relatively specific to the DA (Figure 5B) suggests that it may play an important role in normoxic DA constriction. The function of RhoB in the ductus remains unknown and merits further study. The present report adds to the interest in RhoB as a potential mediator of DA closure, particularly because RhoB gene expression has been suggested to be developmentally regulated in rat DA.\(^\text{20}\)

Although we find an important role for Rho-kinase in DA constriction, the largest proportion of DA constriction depends on calcium influx via the L-type calcium channel, as reported previously.\(^\text{4,8,12}\) This conclusion differs from that of Keck et al,\(^\text{18}\) who studied calcium homeostasis in sheep DASMCs. They found that \( \text{O}_2 \) increased cytosolic calcium by an initial inositol triphosphate–dependent release of intracellular calcium stores, with subsequent entry of extracellular calcium. They paradoxically confirmed that Kv channel blockade mimicked the effects of increasing \( \text{PO}_2 \). Because

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**Figure 8.** Proposed mechanism for \( \text{O}_2 \)-induced activation of the Rho-kinase pathway. Oxygen activates the Rho/Rho-kinase–mediated pathway, which results in phosphorylation of MYPT-1. This phosphorylation inhibits MLCP, thereby increasing the phosphorylation and activity of MLC, which leads to increases in DASMC contraction. Rho-kinase activation by \( \text{O}_2 \) in the DA (but not other fetal arteries) involves a redox-dependent activation of ROCK-1. This likely reflects the relatively unique ability of DA to increase endogenous \( \text{H}_2\text{O}_2 \) in response to increased \( \text{PO}_2 \). Activation of the Rho-kinase pathway rapidly elicits increased expression of pathway components, notably RhoB and ROCK-1. \( \text{O}_2 \)-induced increases in Rho-kinase activity and protein expression are prevented by the ROCK-1 inhibitor.
they did not examine the contribution of SR calcium release to vascular tone, direct comparison to the present study is difficult. However, most of the DA constriction that persists in the absence of extracellular calcium is resistant to cyclopiazonic acid, which argues against a crucial role for SR release in sustaining DA constriction (Figure 3B).

In conclusion, Rho-kinase inhibitors reverse or prevent O2 constriction in human and rabbit DA. O2 and H2O2 activate and induce Rho-kinase. The O2-dependent increase in mitochondrial ROS appears to trigger the activation/induction of Rho-kinase. This mechanism is deficient in preterm rabbit DA.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Persistent (or patent) ductus arteriosus (DA) is one of the most common forms of congenital heart disease, affecting approximately 1 in 2000 live births and 50% of preterm infants. Persistent DA is treated with cyclooxygenase inhibitors, such as indomethacin. In preterm infants, DA closure improves respiratory outcomes and allows earlier hospital discharge. Unfortunately, 20% to 30% of premature neonates fail medical therapy. Functional DA closure is initiated by DA vasoconstriction. This precedes anatomic closure by days and is crucial in the newborn’s transition to air breathing. Prior work has shown that a rise in PO2 initiates DA constriction by increasing mitochondrial reactive O2 species production in DA smooth muscle cells. Increased reactive oxygen species inhibits O2-sensitive K+ channels, which causes membrane depolarization. Depolarization promotes calcium influx, thereby initiating vasoconstriction. In the present report, a new mechanism by which DA constriction is maintained in the absence of calcium influx is revealed. This same PO2-dependent increase in mitochondrial reactive oxygen species activates an enzyme, Rho-kinase, which prolongs the phosphorylation of the contractile apparatus, favoring vasoconstriction. This redox mechanism also increases the expression of Rho-kinase pathway components, further strengthening calcium sensitization. Enhancing DA Rho-kinase activity could be exploited as a means to close the DA when cyclooxygenase inhibitors fail. Conversely, inhibition of Rho-kinase (using specific inhibitors) reverses PO2-induced DA constriction and may have promise in maintaining DA patency as a bridge to congenital heart surgery.
Oxygen Activates the Rho/Rho-Kinase Pathway and Induces RhoB and ROCK-1 Expression in Human and Rabbit Ductus Arteriosus by Increasing Mitochondria-Derived Reactive Oxygen Species: A Newly Recognized Mechanism for Sustaining Ductal Constriction

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