Oxygen-induced Constriction of Rabbit Ductus Arteriosus Occurs via Inhibition of a 4-Aminopyridine-, Voltage-sensitive Potassium Channel

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Abstract

The ductus arteriosus (DA) is a vital fetal structure allowing blood ejected from the right ventricle to bypass the pulmonary circulation in utero. Closure of the ductus arteriosus at birth, essential for postnatal adaptation, is initiated by an increase in oxygen (O\textsubscript{2}) tension. We recently demonstrated the presence of O\textsubscript{2}-sensitive potassium channels in the fetal and adult pulmonary circulation which regulate vascular tone in response to changes in O\textsubscript{2} tension. In this study, we assessed the cellular mechanisms underlying O\textsubscript{2}-induced constriction of the ductus arteriosus in late-gestation fetal rabbits. We report that O\textsubscript{2} reversibly inhibits a 58-pS voltage- and 4-aminopyridine-sensitive potassium channel, causing membrane depolarization, an increase in intracellular calcium through L-type voltage-gated calcium channels, and constriction of the ductus arteriosus. We conclude that the effector mechanism for O\textsubscript{2} sensing in the ductus arteriosus involves the coordinated action of delayed rectifier potassium channels and voltage-gated calcium channels. (J. Clin. Invest. 1996. 98:1959–1965.) Key words: oxygen-sensing • ion channels • fetal physiology • calcium • vascular tone

Introduction

The ductus arteriosus (DA) is a vital fetal structure allowing blood ejected from the right ventricle to bypass the pulmonary circulation in utero. Closure of the DA at birth, essential for postnatal adaptation, is initiated by an increase in oxygen (O\textsubscript{2}) tension (1–4). O\textsubscript{2} exerts a direct constrictor effect on DA vascular smooth muscle (4). Many agents, such as vasoconstrictor prostanoids (5) and endothelin-1 (6, 7), have been proposed to act as O\textsubscript{2} effectors. However, experimental evidence suggests that O\textsubscript{2}-induced ductal constriction is not mediated by a single mediator. Despite extensive investigation, the cellular mechanisms whereby O\textsubscript{2} induces constriction of the DA remain elusive. We recently demonstrated the presence of O\textsubscript{2}-sensitive potassium (K\textsuperscript{+}) channels in the fetal and adult pulmonary circulation which regulate vascular tone in response to changes in O\textsubscript{2} tension (8, 9). We hypothesized that O\textsubscript{2}-induced DA constriction is mediated by inactivation of O\textsubscript{2}-sensitive K\textsuperscript{+} channels. Inhibition of these channels causes membrane depolarization, an increase in intracellular calcium ([Ca\textsuperscript{2+}]), and constriction of the DA. Using late-gestation fetal rabbit DA, we tested the effects of O\textsubscript{2} and K\textsuperscript{+} channel antagonists on tension in isolated DA, on whole cell K\textsuperscript{+} current (I\textsubscript{K}) and membrane potential (E\textsubscript{m}) in DA vascular smooth muscle (VSM) cells and on [Ca\textsuperscript{2+}] levels.

Methods

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication No. 85–23, revised 1985) and was approved by the Minneapolis VA Medical Center Animal Studies Subcommittee and the University of Minnesota Animal Care Committee. Pregnant New Zealand White rabbits at 30 or 31 d of gestation (term = 31 d) were anesthetized with ketamine 75 mg and xylazine 20 mg intramuscularly and 50 mg pentobarbital intravenously. The fetal pups were delivered by cesarean section and a midline sternotomy was performed on the pups, before initiation of respiration. The heart, lungs, and great vessels were excised en bloc and placed in deoxygenated Earle’s solution. The DA was carefully dissected free from adventitia under a dissecting microscope and severed distal to the takeoff of the left pulmonary artery and proximal to the insertion into the descending thoracic aorta.

Tension measurements in isolated DA rings. The isolated DA was placed between two stainless steel wires connected to strain gauge transducers in a 3-ml bath containing Earle’s solution equilibrated with 0% O\textsubscript{2}, 5% CO\textsubscript{2} (referred to as hypoxia, pO\textsubscript{2} = 22 ± 1 Torr, n = 15) or 20% O\textsubscript{2}, 5% CO\textsubscript{2} (normoxia, pO\textsubscript{2} = 133 ± 1 Torr, n = 15) at an optimum tension of 400 mg. The optimum tension was determined by measuring the maximum contractile response to 60 mM KCl at varying basal tensions in hypoxia (200–1,000 mg tested in preliminary experiments). When desired, the endothelium was denuded by repeatedly passing a small wire through the lumen. The absence of endothelium was confirmed by the lack of relaxation to substance P (0.1 μM). Data were recorded using an analog-digital computer system (MacLab, AD Instruments Inc., Medford, MA).

After equilibration in hypoxia for 30 min, the DA was exposed to a normoxic solution and the effect of tension was recorded. The DA was returned to hypoxia and a dose–response was performed to one
of the following K+ channel antagonists: 4-aminopyridine (4-AP; 1, 5, 10 mM), glibenclamide (GLI; 1, 10 μM), or tetraethylammonium (TEA; 1, 5, 10 mM), preferential inhibitors of delayed rectifier (K1, 5 mM), ATP-sensitive (KATP), and Ca2+-activated (KCa) K+ channels, respectively. The DA was exposed to normoxia in the continued presence of K+ channel inhibition to test any additive effect of O2. Each DA was exposed to only one K+ channel antagonist. The number of rings used with each K+ channel blocker is given in the legend to Fig. 1.

In a separate group of DA rings, the role of L- and T-type Ca2+ channels in O2-induced DA constriction was assessed. To eliminate the effects of prostanoids and nitric oxide, the endothelium was denuded as described above and rings were pretreated with indomethacin (3 μM) and L-Nω-nitro arginine methyl ester (L-NAME, 0.1 mM). After constriction with normoxia, the nonspecific Ca2+ channel blocker is given in the legend to Fig. 1.

Whole-cell patch-clamp technique. The effect of O2 and K+ channel blockade on whole-cell IK and Erev was assessed using the amphotericin-perforated patch-clamp technique (11). Freshly dispersed DA VSM were obtained daily by enzymatic digestion with 0.5 mg/ml papain and 0.5 mg/ml collagenase. Cells were perfused (2 ml/min) with a solution containing (mM): NaCl 115, NaHCO3 25, KCl 4.2, MgCl2 0.5, CaCl2 1.5, glucose 10, and Hepes 10, pH 7.4, equilibrated with 0% O2, 5% CO2 (hypoxia, pO2 ~ 33 Torr) or 20% O2, 5% CO2 (normoxia, pO2 ~ 130 Torr) at 32°C. Pipette solutions contained (mM): KCl 140, MgCl2 1.0, EGTA 5, phosphocreatine 2, Heps 10, and amphotericin B 120 μg/ml, pH 7.2. Cells were voltage clamped at a holding potential of −70 mV and currents evoked by 10-mV steps using test pulses of 200 ms in duration at a rate of 0.033–0.1 Hz. To record Erev, cells were held in current clamp at resting Erev. Data were recorded on-line and analyzed using pCLAMP 6.03 software (Axon Instruments, Foster City, CA) as described previously (9).

To determine the pharmacology of whole-cell currents and establish the effects of O2 on the currents, cells were exposed to O2 and K+ channel antagonists. After equilibration in hypoxia, cells were exposed to normoxia (n = 8) for 2–4 min and returned to hypoxia to record recovery currents or Er. Under hypoxic conditions, cells were exposed to 4-AP (1 mM, n = 6), GLI (1 mM, n = 4), or TEA (5 mM, n = 3) while recording IK or Erev. In some cells, more than one K+ channel antagonist was tested.

Cell-attached, single channel recordings. Cell-attached, single channel recordings were performed as described previously (9). Bath and pipette solutions were identical to those described above, except amphotericin was excluded from the pipette solution. Single channel recordings were performed in hypoxia, normoxia, and after return to hypoxia (n = 3). 4-AP (1 mM) or TEA (5 mM) was administered in hypoxia. Channel activities were expressed as NP, where N is the number of channels and P is the probability, measured at a pipette potential of +40 mV.

Measurement of [Ca2+]. For analysis of [Ca2+], isolated DA rings were loaded with the Ca2+-sensitive fluorophore Fura-2 AM (12 μM) at 37°C in room air for 2.5 h. After rinsing, the ring was placed between stainless steel stirrups connected to a force transducer, in a heated bath (37°C) atop an inverted microscope (Nikon Diaphot). Tension was measured exactly as in the previously described ring
Studies. Rings were stimulated with alternating 340- and 380-nm wavelengths at 60 Hz while collecting the 510-nm emission with an epifluorescence microscope and photomultiplier (Photon Technology Inc.) A 75 W xenon lamp served as the light source. [Ca\(^{2+}\)] was calculated using the Grynkiewicz formula (12): [Ca\(^{2+}\)] (nM) = \(K_d \times \frac{R - R_{\text{min}}}{R_{\text{max}} - R}(s_{380}/s_{430})\), where R is the ratio of 340/380 under the experimental condition. R\(_{\text{max}}\) was obtained by permeabilizing the ring with 40 μM ionomycin, thus saturating Fura-2 with extracellular Ca\(^{2+}\). R\(_{\text{min}}\) was determined by chelating all free Ca\(^{2+}\) with 10 mM EGTA. sf380 is the maximum 380-nm intensity obtained and sb380 is the minimum. The experimentally determined dissociation constant (\(K_d\)) for Fura-2 was 245 nM.

After equilibration in hypoxia, the ring was exposed to normoxia (n = 4), while measuring [Ca\(^{2+}\)] and tension. The effect of 10 mM 4-AP was measured in hypoxia (n = 3).

**Data analysis.** The results are presented as the mean±SEM. Inter-group differences were analyzed using the factorial ANOVA with the Fisher least significant post-hoc test. I-V plots were analyzed using repeated measures ANOVA. \(P < 0.05\) was required for significance. Statistical calculations were performed using Statview 4.1 for Macintosh (Abacus Concepts, Berkeley, CA).

**Results**

**Tension measurements in isolated DA rings.** To test the hypothesis that O\(_2\) constricts the DA by inhibiting K\(^+\) channels, we examined the effect of K\(^+\) channel antagonists on tension in isolated DA rings. 4-AP caused a dose-dependent increase in DA tension in endothelium-intact (Fig. 1) and -denuded (Fig. 2) rings in hypoxia, with 10 mM constricting to the same degree as normoxia. Neither GLI nor TEA consistently increased tension in endothelium-intact (Fig. 1) or -denuded DA rings (data not shown). There was little additive constrictor effect of O\(_2\) in the presence of 4-AP, whereas the addition of normoxia in the presence of GLI and TEA caused further constriction (Figs. 1 and 2). These data demonstrate that 4-AP, a preferential K\(_{\text{ATP}}\) inhibitor, constricts the DA, acting independently of the endothelium, and that O\(_2\) exerts no additional constrictor effect in the presence of 4-AP.

**Whole-cell patch–clamp technique.** The effect of O\(_2\) on whole-cell I\(_K\) was tested using the amphotericin-perforated patch–clamp technique in DA VSM (Fig. 3). Normoxia reversibly suppressed I\(_K\) by 22.5±2.7% (+40 mV, n = 9). 4-AP (1 mM) and TEA (5 mM) administered in hypoxia inhibited I\(_K\) 65.3±6.3% and 50.3±9.0% (+40 mV), respectively, whereas GLI (10 μM) was without effect. TEA suppressed I\(_K\) at test potentials positive to resting E\(_m\), whereas O\(_2\) suppressed I\(_K\) at potentials near resting E\(_m\) (Fig. 3 B, inset). This explains why O\(_2\) causes E\(_m\) depolarization and DA constriction, while TEA causes neither depolarization nor vasoconstriction. The effects of O\(_2\) and K\(^+\) channel blockade on E\(_m\) are demonstrated in Fig. 3 C. Resting E\(_m\) in hypoxic DA VSM was −34.3±2.1 mV (n = 13) and depolarized to −19.8±5.2 mV within 2 min of normoxia (n = 4, \(P < 0.05\)). 4-AP in hypoxia depolarized E\(_m\) to a similar degree as normoxia. GLI and TEA did not alter E\(_m\). The addition of normoxia in the presence of 4-AP did not cause further E\(_m\) depolarization (data not shown), implying that O\(_2\) and 4-AP may act through a similar mechanism.

**Cell-attached, single channel recordings.** To further characterize the K\(^+\) channel inhibited by O\(_2\), cell-attached, single channel recordings were performed (Fig. 4). Normoxia inhibited a K\(^+\) channel with a calculated conductance of 58±1 pS (n = 3), with an associated decrease in NP\(_m\) of 74%. This channel was inhibited by O\(_2\) and 4-AP, but not TEA. These data suggest that O\(_2\)-induced DA constriction is mediated through inhibition of a specific K\(_{\text{ATP}}\) channel, resulting in membrane depolarization. Normoxia did not inhibit a 150-pS channel, which was blocked by TEA.

**Measurement of [Ca\(^{2+}\)], and role of Ca\(^{2+}\) channels.** The effect of O\(_2\) on [Ca\(^{2+}\)], in isolated DA rings is demonstrated in Fig. 5 A. Normoxia increased [Ca\(^{2+}\)] 258±90%, while simultaneously increasing tension 180±34%. The role of extracellular Ca\(^{2+}\) and voltage-gated Ca\(^{2+}\) channels in O\(_2\)-induced DA tone is demonstrated in Fig. 5 B. La\(^{3+}\) and NIS produced profound vasoconstriction in O\(_2\)-constricted DA rings, while RO40-5967 and VEH were without significant effect. These data suggest that O\(_2\)-induced DA constriction is primarily dependent upon Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels.

**Figure 2.** 4-AP increases tension in endothelium-intact and -denuded DA rings. 4-AP caused a dose-dependent increase in tension under hypoxic conditions in both endothelium-intact (n = 15) and -denuded rings (n = 5). The addition of normoxia did not increase tone beyond that observed with 10 mM 4-AP. \(*P < 0.05\) compared with baseline (BL).
Discussion

Constriction of the DA at birth routes blood flow through the pulmonary circulation, where postnatal gas exchange occurs. Failure of the DA to constrict (patent DA) is a cause of significant morbidity and mortality in premature infants. The increase in O\textsubscript{2} tension at birth exerts a direct effect on DA VSM to initiate DA constriction (4). However, the precise mechanism of O\textsubscript{2}-induced DA constriction remains unknown. This study shows that O\textsubscript{2} inhibits a 58-pS, K\textsubscript{DR} channel in DA VSM, resulting in membrane depolarization, activation of L-type Ca\textsuperscript{2+} channels, and DA constriction.

This is the first complete characterization of the cellular pathway initiated by O\textsubscript{2} and leading to DA constriction. O\textsubscript{2}-sensitive K\textsuperscript{+} channels, initially described in carotid body chemoreceptors (13), also regulate vascular tone in response to changes in pO\textsubscript{2} in pulmonary (8, 9, 14), coronary (15), and cerebral (16) arteries. In fetal resistance pulmonary artery VSM, hypoxia inhibits K\textsubscript{Ca} channels which causes membrane depolarization, activation of voltage-operated Ca\textsuperscript{2+} channels, and pulmonary vasoconstriction (8, 17). In the adult pulmonary circulation, hypoxia inhibits a K\textsubscript{DR} channel and initiates vasoconstriction (9, 18). In the DA, hypoxia activates rather than inhibits K\textsubscript{DR} channels. It is intriguing that subsets of the same class of K\textsuperscript{+} channel (delayed rectifier) respond in an opposite fashion when faced with similar O\textsubscript{2} tensions. Although the two channels appear different based on their different conductances, a molecular approach is necessary to definitively identify the K\textsuperscript{+} channels in each of these O\textsubscript{2}-sensitive tissues. The K\textsubscript{DR} antagonist 4-AP constricts both the pulmonary artery and the DA, suggesting that the O\textsubscript{2} sensor mechanism is proximal to the channel; that is, the channel itself is not the sensor, but perhaps the sensor is an associated subunit which regulates gating of the channel in response to changes in pO\textsubscript{2}. In the pulmonary artery, the sensor appears to be redox modulated, in that hypoxia and reductants close, while normoxia and oxidants open, K\textsuperscript{+} channels (19, 20). The opposing effects of O\textsubscript{2} on the K\textsubscript{DR} channel may be explained by differential modula-

Figure 3. Effect of O\textsubscript{2} and K\textsuperscript{+} channel blockade on whole-cell I\textsubscript{K} and E\textsubscript{m} (A) Representative current traces of I\textsubscript{K} recorded in hypoxia, normoxia, before return to hypoxia (recovery), and after 1 mM 4-AP added in hypoxia. (B) Average current voltage (I–V) relationships for O\textsubscript{2} and K\textsuperscript{+} channel antagonists. Current was normalized to peak I\textsubscript{K} at +50 mV. O\textsubscript{2} (n = 8), 4-AP (1 mM, n = 6), and TEA (5 mM, n = 3) suppressed I\textsubscript{K}, while GLI (10 μM, n = 4) was without effect. (Shaded inset) Average raw currents evoked at −30 mV test potential in hypoxic control (solid bar) and during normoxia or TEA (cross-hatched bar). Normoxia, but not TEA, suppressed I\textsubscript{K} at test potentials near resting E\textsubscript{m}. (C) O\textsubscript{2} (n = 5) and 4-AP (n = 5) depolarized E\textsubscript{m}, while GLI (n = 3) and TEA (n = 3) were without effect. *P < 0.05 compared with control I–V curve, using repeated measures ANOVA. †P < 0.05 compared with hypoxic control. K\textsuperscript{+} channel antagonists were tested in continued hypoxia.
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tion of the channel by cellular or membrane redox state, by differential responses of the pulmonary artery and DA to NAD(P)H oxidase or O$_2$ radicals. We also report the presence of a large conductance, TEA-sensitive channel in the DA which is not inhibited by changes in pO$_2$. The role of this channel was not investigated.

Similar to resistance pulmonary arteries (9), this study suggests that basal E$_{m}$ in the DA is largely controlled by K$_{DR}$ activity. 4-AP depolarized E$_{m}$ and constricted the isolated DA. O$_2$-induced E$_{m}$ depolarization was first reported in 1981 by Roulet and Coburn (21), using microelectrode puncture studies in isolated DA strips. The present study demonstrates that O$_2$ depolarizes E$_{m}$ by suppressing whole-cell I$_K$ and specifically, by decreasing the NP$_O$ of K$_{DR}$ channels. As gating of Ca$^{2+}$ channels is strongly dependent upon E$_{m}$, depolarization activates Ca$^{2+}$ channels, causing an influx of Ca$^{2+}$ and vasoconstriction (22). Nakanishi et al. (23) found that O$_2$ increased [Ca$^{2+}$], in the presence of extracellular Ca$^{2+}$, underscoring the importance of extracellular Ca$^{2+}$ in O$_2$-induced DA constriction. We confirm this observation and show that the Ca$^{2+}$ influx initiated by O$_2$ is carried almost exclusively through L-type Ca$^{2+}$ channels.

Contrary to our findings, Nakanishi et al. (23) reported that 4 µM GLI produced maximal constriction in the isolated DA. They proposed that hypoxia activates K$_{ATP}$ channels, promoting vasorelaxation and that normoxia inhibits K$_{ATP}$ channels to cause vasoconstriction, although the patch-clamp technique was not used to confirm this hypothesis. A similar mechanism, involving K$_{ATP}$ channels, is postulated to mediate hypoxia-induced coronary artery vasodilation (15). In our study, 10 mM 4-AP, but not 10 µM GLI, caused maximal constriction in hypoxia in the majority of rings tested. Furthermore, GLI did not depolarize resting E$_{m}$ and did not suppress I$_K$ in DA VSM. In support of the K$_{DR}$ channel as the O$_2$-sensitive K$^+$ channel subtype, 4-AP depolarized basal E$_{m}$ and suppressed outward I$_K$, as did normoxia. Finally, as O$_2$ inhibits a 4-AP-sensitive, 58-pS channel, this suggests that the O$_2$-sensitive K$^+$ channel belongs to the K$_{DR}$ class.

How do the various mechanisms controlling tone in the DA interact? DA tone is determined by a balance between vasodilator and vasoconstrictor mechanisms. In utero, DA patency is primarily dependent upon production of prostaglandin E2 (24) with a minor contribution by nitric oxide (25). Although there is a decrease in the production and potency of prostaglandin E2 at birth (26), closure of the DA is largely the result of an independent, vasoconstrictor mechanism. The proposed mechanisms by which this vasoconstriction occurs involve O$_2$-induced increases in the production of endothelin-1 (7) or K$^+$ channel inhibition.

Endothelin-1 is a potent constrictor of DA rings (although

Figure 4. The effect of O$_2$ and K$^+$ channel antagonists on single channels. (A) Recordings from a cell-attached patch (+40 mV) demonstrating the presence of two distinct channels, a 58-pS channel, sensitive to 4-AP (1 mM), and a 150-pS channel, sensitive to TEA (5 mM). Normoxia decreased the NP$_O$ of the 58-pS channel, while the 150-pS channel was not inhibited by changes in pO$_2$. (B) Events histograms showing normoxic inhibition of the 58-pS channel.
it may not constrict the ductus in vivo (6). Furthermore, endothelin-1 is produced in the smooth muscle layer of the DA in response to increased O2 levels (7). It does appear, however, that the time course of production of endothelin-1 is slower than the ductal constriction to O2. Nonetheless, an endothelin-1 receptor antagonist (BQ123) does attenuate O2-induced DA constriction in lamb (6). Thus, it is likely that the endothelin-1 mechanism is important in closure of the DA (6). How does the endothelin-1 mechanism relate to the K+ channel mechanism? There are data to support the possibility that the two mechanisms are additive. Although Coceani et al. (6) found that BQ123 attenuates O2-induced DA constriction, there was still significant residual constriction to O2, despite administration of an endothelin-1 antagonist. On exposure to O2, particularly at 95% O2, DA rings constricted to roughly 50% of control levels despite the presence of BQ123 (1 μM) (6).

We conclude that O2-induced DA constriction is initiated by inhibition of a 58-pS KTR channel by O2, which causes EK depolarization, activation of L-type Ca2+ channels, and an increase in [Ca2+]. Identification of the specific K+ channel responsible for initiating normoxic ductal closure may facilitate development of therapy for patent DA.

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References