Reactive Oxygen Metabolites Relax the Lamb Ductus Arteriosus by Stimulating Prostaglandin Production

Ronald I. Clyman, Ola D. Saugstad, Françoise Mauray

To determine whether oxygen metabolites can cause ductus relaxation, we used rings of fetal ductus obtained from 36 near-term lambs and measured the effects of the oxygen metabolites generated by the combination of hypoxanthine and xanthine oxidase. The oxygen metabolites produced by hypoxanthine plus xanthine oxidase caused relaxation of the ductus that was inhibited by catalase (hydrogen peroxide scavenger) but not by superoxide dismutase (superoxide anion scavenger). In addition, hypoxanthine plus xanthine oxidase produced a 14-fold increase in prostaglandin (PG) E₂ production with only twofold increase in 6-keto-PGF₁₀ (the stable metabolite of PGI₂). PGE₂ is the most potent relaxant of the ductus arteriosus. The presence of either catalase or indomethacin blocked both the increase in prostaglandin production and the relaxation. We conclude that reactive oxygen metabolites relax the ductus arteriosus and oppose the normal constriction that occurs after birth. However, the vasoactive effects of reactive oxygen metabolites in the ductus appear to be mediated exclusively through the generation of PGE₂. (Circulation Research 1989;64:1-8)

Postnatal regulation of the ductus arteriosus depends on a balance between oxygen-induced contraction and prostaglandin-induced relaxation (for review, see Clyman¹). Although prostaglandin (PG) E₂ is a minor product of prostaglandin production in the lamb ductus arteriosus, the marked sensitivity of the ductus to PGE₂ indicates that PGE₂ may be the most important endogenous prostaglandin to regulate vessel patency.²,³ It has been hypothesized that reactive oxygen metabolites also may play an important role in ductus regulation; lower concentrations of antioxidants in the ductus, compared with those in neighboring vessels (thoracic aorta and pulmonary artery), make it more susceptible to the action of oxygen metabolites.⁴ Numerous sources of oxidants could play a role in regulating the ductus, including mitochondrial electron transport chain components, endoplasmic reticulum and nuclear membrane electron transport chain components, the xanthine dehydrogenase-oxidase system, prostaglandin synthetase and lipoxygenase systems, and reduced nicotinamide adenine dinucleotide phosphate oxidase of neutrophils.⁵,⁶

After birth, the ductus is exposed to a sudden increase in PaO₂, which leads to constriction of the vessel; this constriction produces ischemia of the inner luminal one third of the vessel wall.⁷ Increased PO₂ increases the production of oxygen radicals.⁸ In addition, ischemia and tissue hypoxia can induce reactive oxygen metabolites.⁹,¹⁰,¹¹ During ischemia, there are disturbances in the intramitochondrial electron transport system with release of ubisemiquinone, flavoprotein, and superoxide radicals,⁶,⁹ increases in tissue and circulating hypoxanthine concentrations,¹²,¹³ conversion of xanthine dehydrogenase to oxidase,¹² and large increases in reducing equivalents.¹⁴ Free radical production can proceed at very low oxygen tensions (50% of control values at a PO₂ of 1 mm Hg). Under ischemic conditions, there is a loss of intracellular free radical protective enzymes¹⁵,¹⁶ so that the increased concentrations of free radicals are then free to react with phospholipid membranes and disrupt cellular transport processes. Reactive oxygen metabolites have been found to alter smooth muscle tone in both pulmonary and systemic blood vessels. However, it is impossible to generalize the response from any single vascular bed to others because each bed is susceptible to a different oxygen species and has its own unique con-

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tactile response. In the rabbit lung, reactive oxygen metabolites generated by xanthine oxidase produce vasoconstriction, which is mediated by a product of arachidonic acid cyclooxygenase; in the rat lung, the same generating system produces relaxation, which is independent of cyclooxygenase. In systemic arteries, xanthine oxidase causes relaxation that depends on the production of the hydroxyl, hydrogen peroxide, and superoxide metabolites of oxygen; in pulmonary vessels, the xanthine oxidase response is independent of superoxide anion production. To add to these complexities, in the brain, reactive oxygen metabolites may mediate the effects of activating the cyclooxygenase system rather than vice versa as found in the lung.

Therefore, we have investigated the effects of chemically generated oxygen metabolites on the isolated lamb ductus arteriosus. We have examined the interactions between oxygen metabolite production, oxygen-induced contraction, and PGE2-induced relaxation.

Materials and Methods

Thirty-six late gestation (134±5 days of a 145-day term) fetal lambs were delivered by cesarean section (under maternal low spinal anesthesia) and were killed by rapid exsanguination. The ductus arteriosus was dissected free from loose adventitial tissue and divided into 1-mm thick rings (23±8 mg wet weight, n=36) that were placed in separate 150 ml organ baths. To measure prostaglandin production by the rings of ductus arteriosus, rings were prepared as described above and then placed in 10 ml organ baths. To measure PGE2 and 6-keto-PGF1α (the stable metabolite of PGI2) production by the ductus, the 10 ml bath solution was changed and collected. Initially, the the bath solution was bubbled with 100% N2 (P02 20-30 mm Hg), and the ring was allowed to equilibrate for 60 minutes. The bath solution was changed every 30 minutes. Next, the solution was bubbled with 100% O2. The bath solution was changed and collected every 30 minutes for the next 60 minutes (collection periods 1 and 2). After the second collection period, hypoxanthine 100 μM and xanthine oxidase 0.01 IU/ml were added to the bath solution. The solution was collected once the ductus had developed its maximal relaxation (15-minute collection period—collection period 3). Following this collection, indomethacin 5.6x10^-6 M was added to the bath solution in addition to hypoxanthine and xanthine oxidase. The bath solution was changed every 30 minutes for the next 60 minutes, and indomethacin, hypoxanthine, and xanthine oxidase were added to each change of solution. The bath solution from the 30-minute to 60-minute exposure of the ductus to indomethacin, hypoxanthine, and xanthine oxidase was collected (collection period 4).

To determine the effects of catalase on prostaglandin production, paired rings from the same ductus that were studied above were studied according to the same protocol, except that after the exposure to oxygen alone (collection periods 1 and 2), catalase (110 IU/ml final concentration) or boiled catalase was added to the bath solution in addition to hypoxanthine and xanthine oxidase. The bath solution was collected after 15 minutes (collection period 3).
In another experiment, we studied the effects of increasing concentrations of hypoxanthine on the rate of PGF\(_2\alpha\) production. To measure PGF\(_2\alpha\) production by the ductus, the vessel was bathed in 5 ml of Krebs-Tris solution, which was changed and collected every 30 minutes. Initially, the bath solution was bubbled with 100% \(N_2\) for 60 minutes (P\(_O_2\) 24–32 mm Hg). The bath solution then was bubbled with 100% \(O_2\) for two 30-minute collection periods. Next, hypoxanthine was added to the bath solution. The initial concentration was 100 \(\mu\)M. The solution was changed after 30 minutes; the ring then was exposed to 1 mM hypoxanthine for two 30-minute collection periods. Next, indomethacin 5.6x10\(^{-6}\) M and 1 mM hypoxanthine were added to the bath solution, and the ring was allowed to achieve a new steady state tension over the next 60 minutes.

**Extraction, Chromatographic Separation, and Radioimmunoassay of PGF\(_2\alpha\) and 6-keto-PGF\(_{1\alpha}\)**

The 10 ml collections of solution bathing individual rings of ductus arteriosus were divided into 5 ml aliquots: one aliquot for determination of PGF\(_2\alpha\) and one for determination of 6-keto-PGF\(_{1\alpha}\). The aliquots were mixed with 1,500 disintegrations/min of either \[^{[3]H}\]PGF\(_2\alpha\) or \[^{[3]H}\]6-keto-PGF\(_{1\alpha}\) for calculation of recovery. The aliquots were acidified to pH 3.8 with citric acid. A mixture of cyclohexane:ethyl acetate (1:1) was used to extract PGF\(_2\alpha\), while ethyl acetate alone was used to extract 6-keto-PGF\(_{1\alpha}\). The nitrogen evaporated residues were stored at \(-20^\circ\)C.\(^{24}\) PGF\(_2\alpha\) and 6-keto-PGF\(_{1\alpha}\) were determined by radioimmunoassay after their separation by silicic acid chromatography.\(^{24}\) For each concentration point of the standard curve for either PGF\(_2\alpha\) or 6-keto-PGF\(_{1\alpha}\), we carried a 5 ml blank sample of Krebs-Tris solution plus citric acid through the purification process used for the 5 ml bath samples. Appropriate PGF\(_2\alpha\) or 6-keto-PGF\(_{1\alpha}\) standards were added to each of these evaporated residues. This procedure yielded reproducible standard curves with linear titration from 1 to 250 pg PGF\(_2\alpha\) and from 10 to 500 pg 6-keto-PGF\(_{1\alpha}\). The presence of either catalase, hypoxanthine (1 mM), or indomethacin in the bath solution did not alter the binding characteristics of either the PGF\(_2\alpha\) or 6-keto-PGF\(_{1\alpha}\) antisera.

**Statistics**

Statistical analysis of the data was performed by the paired \(t\) test. When more than one comparison was made, Bonferoni's correction was used. Non-parametric data were compared with a paired sign test. Values are expressed as mean±SD.

**Reagents**

Xanthine oxidase (gr III, buttermilk: 1.1 IU/mg protein), catalase (bovine liver: 11,000 IU/mg protein), superoxide dismutase (bovine liver: 5,500 IU/mg protein), hydrogen peroxide, and hypoxanthine were added directly to the bath solution. Indomethacin was prepared in ethanol (16 mg/ml), and aliquots were added to the bath solution. The final concentration of ethanol in the bath solution was 0.0125%. This concentration of ethanol does not prevent prostaglandin production, \(H_2O_2\)-induced relaxation, or hypoxanthine plus xanthine oxidase-induced relaxation of the ductus arteriosus (data not shown). These chemicals were obtained from Sigma Chemical, St. Louis, Missouri. \[^{[3]H}\]PGF\(_2\alpha\) (170 Ci/mmol) and \[^{[3]H}\]6-keto-PGF\(_{1\alpha}\) (130 Ci/mmol) were obtained from New England Nuclear, Boston, Massachusetts. Antisera to PGF\(_2\alpha\) were obtained from Institut Pasteur, Paris, France, and to 6-keto-PGF\(_{1\alpha}\) from Advanced Magnetics (Seragen), Cambridge, Massachusetts. PGF\(_2\alpha\) and 6-keto-PGF\(_{1\alpha}\) were generously supplied by Dr. J.E. Pike of Upjohn, Kalamazoo, Michigan; they were dissolved in ethanol before use.

**Results**

Rings of lamb ductus arteriosus initially were incubated in a low \(P_O_2\) environment until a steady tension developed (2.07±0.85 gm/mm\(^2\), \(n=36\) lambs). They were then exposed to an increased \(P_O_2\) (680–700 mm Hg). All rings contracted when exposed to the high \(P_O_2\). The "oxygen-induced tension" was considered to be the difference between the steady state tensions at high and low \(P_O_2\) (4.79±1.63 gm/mm\(^2\), \(n=36\)).

A cumulative dose response to increasing concentrations of hypoxanthine was performed in eight oxygen contracted rings (Figure 1). At concentrations of hypoxanthine ≤100 \(\mu\)M, there were minimal effects upon the ductus arteriosus. Concentrations greater than 100 \(\mu\)M were associated with significant relaxation of the ductus.

Similarly, xanthine oxidase (0.01 IU/ml) produced a minimal relaxation of the ductus (Figure 2a). However, when xanthine oxidase was combined with hypoxanthine, there were marked effects upon the ductus arteriosus at high concentrations. Rings of ductus from eight fetal lambs were allowed to reach a steady tension in high \(P_O_2\). Next, hypoxanthine was added in increasing concentrations. Relaxation of the ductus is expressed as the percent inhibition of the oxygen-induced tension prior to the hypoxanthine additions. The oxygen-induced tension was 5.74±1.33 gm/mm\(^2\). Values are mean±SD.
Relaxation decreases in the tension of the oxygen constricted rings (Figure 2a–c). These findings suggested that the ductus relaxation might be due to the reactive oxygen metabolites produced by the hypoxanthine–xanthine oxidase reaction.

To confirm the role of oxygen metabolites in ductus relaxation, we added oxygen metabolite scavengers to the bath solution prior to the introduction of hypoxanthine and xanthine oxidase (Figure 3). Catalase, a scavenger of hydrogen peroxide, inhibited the relaxation caused by both hypoxanthine and xanthine oxidase or by 1 mM hypoxanthine alone. Superoxide dismutase did not prevent either relaxation. Neither superoxide dismutase nor catalase by itself had an effect on ductus arteriosus tension. In addition, hydrogen peroxide produced a dose dependent relaxation of the ductus arteriosus (Figure 4).

From each pair of rings obtained from 21 ductus, we pretreated one ring with indomethacin to see whether the hypoxanthine plus xanthine oxidase relaxation was mediated by prostaglandin production. As we have shown previously,1 rings exposed to oxygen plus indomethacin had a greater increase in tension above the baseline tension at low PO2 (6.8±1.7 gm/mm2) than did rings exposed to oxygen alone (5.3±1.6 gm/mm2, n=21, p<0.001). Indomethacin prevented hypoxanthine plus xanthine oxidase from relaxing the ductus (Figure 5). In addition, indomethacin was able to reverse the ductus relaxation once it had been produced by hypoxanthine plus xanthine oxidase. When hypoxanthine (100 μM) plus xanthine oxidase (0.01 IU/ml) were added to rings from 11 ductus that had previously been constricted by oxygen, the tension dropped to 63±23% of the initial "oxygen-induced tension." When indomethacin 5.6x 10^-6 M was added to the bath solution containing hypoxanthine plus xanthine oxidase, the tension increased to 112±13% of the initial oxygen-induced tension (p<0.01 indomethacin-induced tension versus initial oxygen-induced tension). Similarly, indomethacin blocked the H2O2-induced relaxation of the ductus arteriosus (Figure 4).

Addition of hypoxanthine plus xanthine oxidase also caused a 14-fold increase in PGE2 production.
Our studies show that chemically generated oxygen metabolites can produce relaxation of the ductus arteriosus and inhibit oxygen-induced contraction. To test the effects of oxygen metabolites, we used the hypoxanthine–xanthine oxidase reaction because it has been well characterized and previously used for this purpose. This reaction results in both univalent and divalent reductions of oxygen yielding approximately 20% superoxide anion (O$_2^-$) and 80% H$_2$O$_2$, respectively. In addition, the xanthine oxidase system, in the presence of suitable catalysts, can mediate the formation of an oxidant (or oxidants) more powerful than either O$_2^-$ or H$_2$O$_2$ alone, which, for convenience, has been "called" hydroxyl radical (OH$^-$).

Neither hypoxanthine, in concentrations up to 100 µM, nor xanthine oxidase (0.01 IU/ml) produced a substantial effect on ductus arteriosus tone. When both were combined, there was a significant reduction in tension. This appeared to be mediated by H$_2$O$_2$ or H$_2$O$_2$-derived products such as OH$, but not by O$_2^-$, since catalase (an H$_2$O$_2$ scavenger) but not superoxide dismutase blocked the relaxation. In addition, H$_2$O$_2$ relaxed the constricted ductus when added directly to the organ bath.

In our studies, catalase by itself had no effect on the oxygen-contracted ductus arteriosus. Its inability to produce further contraction of the oxygen-constricted ductus suggests that under these experimental conditions, H$_2$O$_2$ was not being produced by the ductus in amounts that would affect the vessel’s tone, or that catalase could not get to the intracellular sites where H$_2$O$_2$ was being produced.

There are numerous ways that reactive oxygen metabolites might affect blood flow. Neerman et al have suggested that the "lipid triad" composed of lipid peroxidation, activation of lipases and phospholipases, and the production of free fatty acids and lyso-phospholipids that mediates oxygen radical effects. Lipid peroxidation leads to an increase in membrane fluidity and permeability.

**Discussion**

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Figure 6. Effects of catalase and indomethacin on the hypoxanthine (Hx) plus xanthine oxidase (XO)-induced generation of PGE2 and 6-keto-PGF1α as well as the relaxation of the ductus arteriosus. Paired rings of ductus from seven lambs were suspended in 10 ml organ baths as described in “Materials and Methods.” The ductus rings were exposed to (in the following order): N2, O2, O2 plus hypoxanthine (100 μM) plus xanthine oxidase (0.01 IU/ml), and O2 plus hypoxanthine (100 μM) plus xanthine oxidase (0.01 IU/ml) plus indomethacin (5.6×10⁻⁶ M). To one of the rings, catalase (110 IU/ml) was added in addition to the hypoxanthine plus xanthine oxidase and in addition to the hypoxanthine plus xanthine oxidase plus indomethacin. To the other ring, boiled catalase was added at the same time points. The bath solution was collected for measurement of PGE2 and 6-keto-PGF1α after 30 minutes of ductus exposure at each condition; the collection period for hypoxanthine plus xanthine oxidase (with or without active catalase) was 15 minutes. The tension in the ductus was measured at the end of each collection period. Prostaglandins are expressed as median values of pg released/mg wet weight/collection period. Tension is expressed as mean±SD. Comparing the values obtained when the ductus was exposed to Hx+XO versus the prior period in O2 alone: *p<0.02, **p<0.002 (n=7). Comparing the values obtained when the ductus was exposed to Hx+XO+indomethacin versus Hx+XO: tp<0.02, ††p<0.005 (n=7).

Figure 7. Effects of hypoxanthine (Hx) on ductus relaxation and PGE2 production. Rings of ductus from six lambs were suspended in 5 ml organ baths as described in “Materials and Methods.” The bath solution conditions were changed every 30 minutes. The ductus rings were exposed to (in the following order): N2, O2, O2, O2 plus hypoxanthine (100 μM), O2 plus hypoxanthine (1,000 μM), O2 plus hypoxanthine (1,000 μM), O2 plus hypoxanthine (1,000 μM) plus indomethacin (5.6×10⁻⁶ M). The bath solution was collected every 30 min for measurement of PGE2. The 30-minute collection period for the indomethacin plus hypoxanthine exposure was begun after the ductus had already been exposed to indomethacin and hypoxanthine for 30 minutes. The tension in the ductus was measured at the end of each collection period. PGE2 values are expressed as picograms released per milligram wet weight per 30 minute collection period. Values obtained when the ductus was exposed to oxygen versus oxygen plus hypoxanthine (1,000 μM): *p<0.01, n=6. Values obtained when the ductus was exposed to hypoxanthine (1,000 μM) versus hypoxanthine (1,000 μM) plus indomethacin: †p<0.01, n=6.

Aldehydes formed during the peroxidation of fatty acids can cross-link primary amine groups.6 Oxidation of sulfhydryl groups in the calcium-ATPases of plasma membranes, mitochondria, and endoplasmic reticulum decreases their activity.28,29 Oxygen metabolites can also damage Na, K-ATPase.30 In addition to its ability to alter protein structure, oxidation reactions can accelerate both selective proteolysis and enzymatic function.6 For example, hydrogen peroxide stimulates the activity of guanylate cyclase, thus increasing cyclic GMP and causing relaxation.31 Oxygen-derived metabolites can either stimulate or depress endothelium-dependent relaxations.32 In addition, there are complex interactions between reactive oxygen metabolites and prostaglandin production. During the activation of cyclooxygenase, OH* is formed. The hydroxyl radical in turn inactivates the prostaglandin synthetase.33 On the other hand, active oxygen species can liberate arachidonic acid34,35 by stimulating lipid peroxidation of cell membranes5-34 and activating phospholipase A2.35 Oxygenases that catalyze the first committed step in eicosanoid formation have an unusual requirement for lipid hydroperoxides.36 Both H2O2 and lipid hydroperoxides can accelerate cyclooxygenase activity.38

Our studies show that xanthine oxidase–derived oxygen metabolites can stimulate the production of PGE2 and PGI2 in isolated lamb ductus arteriosus. The relation between oxygen metabolite–induced
PGE₂ production and vasodilation seems likely. Although basal PGE₂ production is approximately 1/50 of PG₁ production (reflected by its stable metabolite 6-keto-PGF₁α), prior studies have shown that PGE₂ is approximately 1,000 times more powerful than PG₁ in relaxing ductus constriction.² ³ In addition, xanthine oxidase–derived oxygen metabolites induce a 15-fold increase in PGE₂ but only a doubling of PG₁. Addition of catalase completely inhibited the hypoxanthine–xanthine oxidase–induced prostaglandin production and blocked the associated relaxation of the ductus. Furthermore, indomethacin blocked both the H₂O₂ and the hypoxanthine–xanthine oxidase–induced relaxation of the ductus; in addition, indomethacin reversed the increased rate of prostaglandin production and relaxation that had been previously induced by hypoxanthine–xanthine oxidase. In some studies, indomethacin has been found to be both a weak scavenger and a weak inhibitor of free radical formation; however, under experimental conditions similar to those described in the present study, indomethacin has not been found to interfere with oxygen metabolite production.¹⁷ These results suggest that PGE₂ was the mediator of oxidant-induced vasodilation of the ductus arteriosus.

It is interesting to note that H₂O₂ or H₂O₂-derived metabolites produce opposite effects in the rabbit pulmonary circulation, namely, vasoconstriction that can be inhibited by catalase and indomethacin.¹⁷ ²² The difference in the response of these two neighboring vascular beds is probably due both to differences in the local production of prostaglandins as well as to differences in the sensitivity of the vessels to the prostaglandins produced. For example, the ductus arteriosus is exquisitely sensitive to the vasodilating action of PGE₂ but insensitive to the vasoconstricting action of thromboxane A₂; on the other hand, the pulmonary circulation is relatively insensitive to the vasodilating effects of PGE₂ but sensitive to the vasoconstricting action of thromboxane A₂.¹⁷ ³⁰

We found that hypoxanthine was able to relax the ductus by itself when administered in high concentrations. Concentrations greater than 100 μM produced significant relaxation; these levels are achieved during ischemia in other tissues.¹² ¹³ These high concentrations of hypoxanthine produced relaxation that was partially preventable by catalase (but not by superoxide dismutase—data not shown); this suggests that part of the hypoxanthine-induced relaxation was secondary to H₂O₂ produced by the ductus itself. It is possible that sufficient xanthine oxidase is already present in the ductus (or can be converted from xanthine dehydrogenase) so that oxygen metabolites are produced when hypoxanthine is added, even in the absence of exogenous xanthine oxidase. On the other hand, hypoxanthine, by itself, may stimulate the release of arachidonic acid; by scavenging the free radicals produced during the conversion of PGG₂ to PGH₂, catalase might inhibit the cyclooxygenase activity. Catalase was not as effective in blocking the relaxation caused by high concentrations of hypoxanthine as it was in blocking the relaxation due to the combination of hypoxanthine plus exogenous xanthine oxidase. This may be due to the inability of exogenous catalase to get to the intracellular site where H₂O₂ is produced, or it may be due to an action of hypoxanthine that is independent of the generation of H₂O₂. Hypoxanthine may have a direct effect on the ductus smooth muscle through purine receptors (R.I. Clyman, unpublished results). The relaxation due to high concentrations of hypoxanthine was also associated with increased PGE₂ production. Indomethacin inhibited the increased production of PGE₂ and reversed the relaxation that was previously induced by 1 mM hypoxanthine. In four experiments, pretreatment of the ductus with indomethacin prevented 1 mM hypoxanthine from relaxing the tissue (data not shown).

Thus, both reactive oxygen metabolites and hypoxanthine may relax the ductus and inhibit postnatal constriction. These effects are not independent but rather work through the production of PGE₂. Reactive oxygen metabolites may account for or contribute to the increased PGE₂ production that has been observed when the ductus is exposed to elevated Pₐ.⁴¹ ⁴₂ This PGE₂ opposes the oxygen-induced contraction of the ductus, and the final degree of ductus constriction is determined by the vessel’s sensitivity to PGE₂.

Similarly, after contraction of the ductus arteriosus, there is ischemia of the vessel wall. Generation of hypoxanthine and reactive oxygen metabolites may be responsible for the increased production of PGE₂ found in ischemic ductal tissue. This source of PGE₂ appears to be responsible for reopening of the ductus after initial closure in preterm infants.⁴³

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