Role of Cyclooxygenase-2 in the Generation of Vasoactive Prostanoids in the Rat Pulmonary and Systemic Vascular Beds

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**Background**—Prostanoid synthesis by the cyclooxygenase (COX)-2 pathway plays an important role in inflammation, and recent studies have shown the presence of COX-2 in the normal rat lung. However, the role of COX-2 in the generation of vasoactive prostanoids in the rat is uncertain. In the present study, the hypothesis that synthesis of vasoactive prostanoids via the COX-2 pathway can alter pulmonary and systemic vascular resistance was investigated, and the effects of selective COX-2 inhibitors on pulmonary and systemic responses to the prostanoid precursor arachidonic acid were examined in the anesthetized rat with a recently developed right-heart catheterization technique.

**Methods and Results**—Injections of arachidonic acid caused dose-related increases in pulmonary vascular resistance and decreases in systemic vascular resistance. These responses were attenuated by selective COX-2 inhibitors and a selective COX-1 inhibitor, whereas responses to exogenous prostanoids were not altered. Nimesulide or NS-398 did not alter arachidonic acid–induced platelet aggregation in rat platelet-rich plasma. Western blot analysis and immunostaining showed the expression of both COX isoforms in the rat lung.

**Conclusions**—The results of these experiments suggest that arachidonic acid is converted into vasoactive prostanoids by the COX-2 and COX-1 pathway in the pulmonary and peripheral vascular beds in the rat and that TXA2 is a major prostanoid formed in the normal rat lung. (*Circulation.* 2003;108:896-901.)

**Key Words:** prostaglandins • thromboxane • rat • lung • enzymes

Cyclooxygenase (COX) is the rate-limiting step in the formation of prostanoids from arachidonic acid. Two isoforms of the enzyme have been identified. COX-1 is constitutively expressed and is thought to be involved in physiological processes, whereas COX-2 is an inducible isofrom readily upregulated by inflammatory stimuli. Although COX-2 is generally believed to be inducible, there is evidence that COX-2 is expressed constitutively; however, the expression is usually low under physiological conditions. The cellular localization of COX-1 and COX-2 in the normal rat lung, an organ with high COX activity, has been examined, and the expression of COX-1 and -2 was detected with intense COX-2 staining in smooth muscle of arteries. In studies in the isolated buffer-perfused rat lung, arachidonic acid was converted into vasoactive prostanoids by way of the COX-2 pathway. Although it has been postulated that COX-2 may be involved in physiological processes, there is little information available about the role of COX-2 in the generation of vasoactive prostanoids in the pulmonary and systemic vascular beds in the intact-chest rat. Arachidonic acid is rapidly converted into vasoactive prostanoids in the pulmonary vascular bed in vivo, and responses are attenuated by nonselective COX inhibitors. Coronary vasodilator responses to arachidonic acid in the dog are blocked by a selective COX-2 inhibitor, whereas COX-1 has been shown to be the major isofrom in the generation of vasoactive prostanoids in the cerebral circulation of mice. However, little is known about the role of COX-2 in the generation of vasoactive prostanoids in the rat. The present study was undertaken to investigate the role of COX-2 and COX-1 in the formation of vasoactive prostanoids in the pulmonary and systemic vascular beds of the intact-chest rat.

**Methods**

For hemodynamic studies, pulmonary arterial, systemic, and wedge pressures and cardiac output were measured by a right-heart catheterization procedure. Sprague-Dawley rats weighing 250 to 400 g were anesthetized with Inactin (140 mg/kg IP), with supplemental doses given intravenously as needed to maintain a uniform level of anesthesia. The animals were strapped to a fluoroscopic table. Body temperature was monitored with a rectal probe (Yellow Springs Instruments) and maintained at 37°C with a warming lamp. The trachea was cannulated, and the rats spontaneously breathed room air enriched with 100% O2 or were ventilated with a Harvard model 683 rodent respirator. A femoral artery was catheterized with PE 90 tubing, and pressure was measured with a Statham P23 transducer.
were measured with a Corning model 178 analyzer with a 400-μL blood sample from the femoral artery catheter. All catheter positions were verified at postmortem examination.

Because of the relationship between pressure and flow and the limitation that flow could only be measured at one time point during a response, experiments were performed under constant-flow conditions. A specially designed 3.5F triple-lumen balloon perfusion catheter (Nu-Med) was passed from the left external jugular vein through the right atrium and ventricle into the main pulmonary artery and into the arterial branch of the right lower lung lobe under fluoroscopic guidance. Perfusion pressure was measured from the port at the distal tip of the catheter. The perfusion port was 5 mm from the catheter tip, and the balloon was affixed to the third port just proximal to the perfusion port. When the perfusion catheter is positioned correctly, distension of the balloon will decrease pressure measured from the port at the catheter tip from pulmonary arterial levels to (pump off) wedge pressure values.

After administration of heparin (1000 U/kg IV), a portion of the right lower lung lobe (~50%) is perfused with blood withdrawn from the aorta. Lobar blood flow is maintained at a constant rate with a Masterflex pump (Cole-Parmer Co). The flow rate averaged 15 mL/min, and arachidonic acid injections were made into the lobar perfusion circuit distal to the perfusion pump.

Arachidonic acid sodium salt (Sigma) was dissolved in 0.9% saline, U46619 and prostaglandin E1 (PGE1; Cayman Chemical) were dissolved in 95% ethanol, and dilutions were made in 0.9% saline solution. Norepinephrine hydrochloride, angiotensin II, and ADP (Sigma) were dissolved in 0.9% saline. Nimesulide, NS-398, and SC-560 (Cayman Chemical) were dissolved in 50 mmol/L NaCl, CO2, and daltroban (Smith Kline Beecham) was dissolved in Tris buffer (pH 7.4). Solutions were prepared on a frequent basis. The vehicles for the drugs used in the studies had no significant effect on baseline parameters or on responses to the vasoactive agonists.

Immunohistochemical staining for COX-1 and COX-2 was determined in lung fixed in 10% formalin and paraffin embedded, and sections were deparaffinized in xylens and hydrated through graded alcohols. Endogenous peroxidases were quenched with 3% H2O2, and the sections were washed with PBS. Nonspecific binding of IgG was blocked with normal horse serum (1:50) in 0.1% bovine serum albumin in PBS. The sections were incubated for 2 hours with a rabbit polyclonal antibody for anti-COX-1 and anti-COX-2 (1:100, Santa Cruz Biotechnology), washed, and incubated for an additional 30 minutes with a biotinylated secondary antibody. After a 30-minute incubation with ABC horseradish peroxidase (DAKO), the substrate (DAB, Vectastain, Vector Laboratories) was added for 5 minutes. This resulted in positive cells that were stained brown. Sections were then stained with hematoxylin, and sections were stained for COX-1 and COX-2 were examined by several observers.

For Western blots, tissue was homogenized (Polytron, Brinkmann Instruments) in ice-cold buffer (HEPES 5 mmol/L, pH 7.9, glycerol 26%, MgCl2 1.5 mmol/L, EDTA 0.2 mmol/L, DTT 0.5 mmol/L, PMSF 0.5 mmol/L) with NaCl (300 mmol/L) and incubated on ice for 30 minutes. After centrifugation twice at 15 000g and 4°C for 20 minutes, protein concentration was determined. For Western blot analysis, the supernatant was mixed with an equal volume of 2% SDS/1% β-mercaptoethanol and fractionated with 8% SDS/PAGE (70 μg/lane). Proteins were then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Sciences) by semidry electroblotting for 1 hour. The membranes were blocked for 1 hour at room temperature with blotto-Tween (5% nonfat dry milk, 0.1% Tween 20) and incubated with a primary polyclonal rabbit anti-COX-1 and primary-COX-2 antibody (1:5000; Santa Cruz Biotechnology). Bound antibody was detected with labeled rabbit anti-rabbit IgG secondary antibody (1:20 000; Santa Cruz Biotechnology) and visualized with enhanced chemiluminescence.

Platelet aggregation was performed in platelet-rich plasma from blood withdrawn from a femoral artery of control rats and rats treated with nimesulide or NS-398 (3 mg/kg IV), sodium mECFenamate (2.5 mg/kg IV), or SC-560 (10 mg/kg IV). The blood was withdrawn into tubes and mixed with 1/10 volume 2.2% trisodium citrate and centrifuged at 500 rpm for 5 minutes. Platelet-rich plasma (0.5 mL)
was placed in the cuvette of a Chrono-Log model 440-VS dual-channel aggregator and stirred at 500 rpm, and aggregation was induced by the addition of arachidonic acid (0.5 mmol/L) or ADP (10 μmol/L).

Data are expressed as mean ± SE and were analyzed with a paired t test or 1-way ANOVA with repeated measures and Scheffé’s F test. A probability value of less than 0.05 was used as the criterion for statistical significance.

Results

Immunohistochemical and Western Blot Analysis

COX-1 and COX-2 protein expression was measured in rat lung, and these data are shown in Figure 1A. Western blot analysis with COX-1 (70 kDa) and COX-2 (72 kDa) specific antibodies detected both COX-1 and COX-2 protein in the rat lung (Figure 1A). The expression of COX-1 and COX-2 in the rat lung was studied by immunohistochemical analysis. Positive immunostaining for COX-1 and COX-2 was detected in normal rat lung, and immunostaining was observed in bronchial epithelial cells, endothelial and smooth muscle cells of small pulmonary arteries, bronchial smooth muscle cells, and lung parenchyma (Figure 1B).

Responses to Arachidonic Acid

Responses to administration of arachidonic acid were investigated, and injections in doses of 0.3 to 3 mg/kg IV caused dose-related increases in pulmonary arterial pressure and decreases in systemic arterial pressure (Figure 2). Cardiac output measured at the peak of pulmonary pressor response and pulmonary wedge pressure were not changed significantly; pulmonary vascular resistance was increased, and systemic vascular resistance was decreased. After administration of the COX-2 inhibitor nimesulide (3 mg/kg IV), the increases in pulmonary arterial pressure and decreases in systemic arterial pressure in response to arachidonic acid were reduced significantly (Figure 2A). The effects of nimesulide on responses to preformed prostaglandins were also investigated, and after administration of nimesulide, changes in pulmonary and systemic arterial pressure in response to intravenous injections of PGE1 and U46619 were not changed (data not shown). Nimesulide had no consistent effect on pulmonary arterial and wedge pressures or on cardiac output but caused a small decrease in systemic arterial pressure. The effects of nimesulide on pulmonary vasoconstrictor responses to arachidonic acid were investigated under constant-flow conditions, and responses were decreased significantly after administration of nimesulide (Figure 2, B and C).

The effect of the selective COX-2 inhibitor NS-398 on responses to arachidonic acid was investigated, and after administration of NS-398 (3 mg/kg IV), increases in pulmonary arterial pressure and decreases in systemic arterial pressure were reduced significantly (Figure 3A).
Effect of Daltroban

After administration of the thromboxane A2 (TXA2) receptor antagonist daltroban (5 mg/kg IV), the increases in pulmonary arterial pressure in response to arachidonic acid were blocked, and a slowly developing decrease in pulmonary arterial pressure was unmasked (Figure 3B). The decrease in systemic arterial pressure was not changed by daltroban (Figure 3B). Cardiac output and pulmonary arterial wedge pressure were unchanged, and the slowly developing decrease in pulmonary arterial pressure in the daltroban-treated animal was attenuated by nimesulide (Figure 3B). Daltroban significantly attenuated the increase in pulmonary and systemic arterial pressure in response to intravenous injections of the TXA2 mimic U46619 but did not alter decreases in pressure in response to PGE1 (Figure 4). Daltroban did not alter responses to angiotensin II or norepinephrine. Daltroban had no significant effect on systemic or pulmonary arterial pressure or on cardiac output. The effect of the COX-1 inhibitor SC-560 on responses to arachidonic acid was assessed, and these data are summarized in Figure 5A. After administration of SC-560 (10 mg/kg IV), increases in pulmonary arterial pressure and decreases in systemic arterial pressure in response to arachidonic acid were decreased significantly (Figure 5A).

Platelet Aggregation Studies

The effect of nimesulide on arachidonic acid–induced platelet aggregation was investigated in plasma from rats treated with nimesulide, NS-398, SC-560, or sodium meclofenamate, and these results are summarized in Figure 5B. The addition of arachidonic acid (0.5 mmol/L) caused a 75% increase in light transmission (percent aggregation) in platelet-rich plasma from control rats (Figure 5B). In platelet-rich plasma from rats treated with nimesulide or NS-398 (3 mg/kg IV) 30 to 60 minutes earlier, arachidonic acid caused an increase in light transmission that was not different from the increase measured in platelet-rich plasma from control animals (Figure 5B). In platelet-rich plasma from rats treated with sodium meclofenamate (2.5 mg/kg IV), a nonselective COX inhibitor, and SC-560 (10 mg/kg IV), a COX-1 inhibitor, the aggregatory response to arachidonic acid was markedly inhibited (Figure 5B). The response to arachidonic acid was not altered when nimesulide or NS-398 was added to the cuvette in concentrations of 3 to 10 μmol/L, and the increase in light transmission (platelet aggregation) induced by ADP (10 μmol/L) was not changed significantly by nimesulide, NS-398, SC-560, or sodium meclofenamate (data not shown).

Discussion

The present results provide evidence in support of the hypothesis that formation of vasoactive prostanoids occurs by way of the COX-2 pathway.14,15 Although it is generally believed that COX-2 is an inducible isoform, it has been reported that both COX isoforms are present in normal rat lung.14 The localization and mRNA expression have been studied, and the expression of COX-1 and -2 was demonstrated with COX-2 expression in smooth muscle of arteries and veins.14 It has been shown in the isolated perfused rat lung that arachidonic acid is converted into vasoactive pro-
stanioids by way of the COX-2 pathway. In the present study, Western blot analysis showed the expression of COX-1 and COX-2, and immunohistochemical studies revealed widespread staining for both COX isoforms in the normal rat lung. The present results show that the selective COX-2 inhibitors nimesulide and NS-398 attenuated the increase in pulmonary arterial pressure and the decrease in systemic arterial pressure in response to arachidonic acid. These data suggest that pulmonary pressor and systemic depressor responses are mediated by the formation of prostanoids in the COX-2 pathway. The selectivity of nimesulide and NS-398 for COX-2 has been documented in the literature and was examined with a platelet aggregation assay. Platelets are reported to have only COX-1, and COX-2 inhibitors do not alter arachidonic acid–induced platelet aggregation. In the present study, arachidonic acid–induced platelet aggregation was blocked in platelet-rich plasma from rats treated with the nonselective COX inhibitor sodium meclofenamate or the selective COX-1 inhibitor SC-560. The mechanism by which COX-2 prostanoids increase pulmonary arterial pressure was investigated, and after treatment with a thromboxane receptor antagonist, the pulmonary pressor response to arachidonic acid was blocked, and a slowly developing decrease in pressure was unmasked. This depressor response was attenuated by nimesulide. These data suggest that the pressor response is mediated by TXA₂ and that after thromboxane receptor blockade, a response mediated by a vasodilator prostanoid was unmasked. These data suggest that TXA₂ and vasodilator prostanoids are formed in the normal lung in vivo via the COX-2 pathway but that a prostanoid-mediated vasodilator response can only be detected after thromboxane receptor blockade. The observation that the pulmonary vasoconstrictor response was attenuated by COX-2 inhibitors suggests that TXA₂ was generated by way of the COX-2 pathway in the lung and that platelets do not play a role. The observation that the COX-2–mediated systemic vasodilator response to arachidonic acid was not altered by daltroban suggests that little if any TXA₂ is formed in the systemic bed and is in agreement with results in the dog coronary vascular bed, in which vasodilator responses to arachidonic acid were attenuated by a COX-2 inhibitor, whereas a thromboxane inhibitor had no effect.

Changes in pulmonary vascular resistance could only be measured at one time point during a response, and because of the relationship between pressure and flow, responses to arachidonic acid and the effects of nimesulide were investi-
gated under constant-flow conditions, which provide a more accurate assessment of pulmonary vascular resistance. In these experiments, injections of arachidonic acid into the perfused lobar artery increased lobar arterial pressure without altering pump-off wedge pressure. The pulmonary lobar vasoconstrictor response to arachidonic acid was significantly reduced by nimesulide, which provides additional support for the hypothesis that vasoconstrictor prostanoids are formed by COX-2 within the rat lung.

Prostanoid formation can proceed by way of the COX-1 or COX-2 pathway, and it has been reported that COX-1 and COX-2 are constitutively expressed in the normal rat lung and in other tissues. In the present study, Western blot analysis showed the presence of COX-1 and -2 protein, and immunostaining for both isoforms was detected in the rat lung. These results, along with hemodynamic studies, suggest that vasoactive prostanoids are formed by the COX-2 pathway in the rat and extend the results of studies demonstrating that vasoactive prostanoids are formed by the COX-2 pathway in the isolated perfused rat lung.

The observation that the pulmonary vasoconstrictor response to arachidonic acid is inhibited by nimesulide, NS-398, and a thromboxane receptor antagonist suggests that TXA$_2$ is a major product formed by the COX-2 pathway in the normal lung. The finding that the response is blocked by COX-2 inhibitors in a dose that did not alter the aggregatory response to arachidonic acid suggests a role for lung cells but not platelets in the formation of TXA$_2$.

The effects of a selective COX-1 inhibitor on responses to arachidonic acid were also investigated, and after administration of SC-560, pulmonary pressor and systemic vasodepressor responses were significantly reduced. These results suggest that formation of vasoactive prostanoids can also occur by way of the COX-1 pathway in the rat.

In conclusion, the present results demonstrate that COX-2 selective inhibitors attenuate pulmonary vasoconstrictor and systemic vasodilator responses to arachidonic acid, whereas responses to exogenous prostanoids are not altered. The COX-2 inhibitors did not alter the platelet aggregatory response to arachidonic acid. These data, along with immunohistological and Western blot analysis, suggest that vasoactive prostanoid formation can occur by way of the COX-2 pathway in the rat. The observation that a thromboxane receptor antagonist blocked the pulmonary vasoconstrictor response to arachidonic acid suggests that TXA$_2$ is a major product of the COX-2 pathway within the pulmonary vascular bed. Studies with the COX-1 inhibitor SC-560 indicate that formation of vasoactive prostanoids also occurs by way of the COX-1 pathway. These data suggest that COX-2 and COX-1 both play a role in the physiological regulation of cardiovascular and pulmonary responses in the normal rat, responses that previously were thought to be mainly mediated by COX-1.

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References

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