Effects of Selective Cyclooxygenase-2 Inhibition on Vascular Responses and Thrombosis in Canine Coronary Arteries

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Background—Prostanoid synthesis via the action of cyclooxygenase-2 (COX-2) is a component of the inflammatory response. Prostacyclin, a product of COX-2 in vascular endothelium, has important physiological roles, such as increasing blood flow to injured tissues, reducing leukocyte adherence, and inhibiting platelet aggregation. We examined the possibility that selective COX-2 inhibition could suppress the protective effects of prostacyclin, resulting in an alteration of the hemostatic balance and vascular tone.

Methods and Results—Circumflex coronary artery thrombosis was induced in dogs by vascular electrolytic injury. Orally administered celecoxib (COX-2 inhibition) or high-dose aspirin (HDA) (COX-1 and COX-2 inhibition) did not alter time to occlusive thrombus formation compared with controls (celecoxib 77.7 ± 7.2 minutes, HDA 72.0 ± 18.5 minutes, control 93.0 ± 21.8 minutes). Oral HDA with an endothelial recovery period (HDA-ER) (COX-1 inhibition) produced a significant increase in time to vessel occlusion (257.0 ± 41.6 minutes). The observed increase in time to occlusion was abolished when celecoxib was administered to animals dosed with HDA-ER (80.7 ± 20.6 minutes). The vasomotor effect of endothelium-derived prostacyclin was examined by monitoring coronary flow during intracoronary administration of arachidonic acid or acetylcholine. In celecoxib-treated animals, vasodilation in response to arachidonic acid was reduced significantly compared with controls.

Conclusions—The results indicate important physiological roles for COX-2–derived prostacyclin and raise concerns regarding an increased risk of acute vascular events in patients receiving COX-2 inhibitors. The risk may be increased in individuals with underlying inflammatory disorders, including coronary artery disease. (Circulation. 2001;104:820-825.)

Key Words: cyclooxygenase ■ prostaglandins ■ aspirin ■ thrombosis

Prostaglandins (PGs) regulate important physiological functions, including gastrointestinal motility, vascular tone, mucosal protection, and immune responsiveness.1 Cyclooxygenases (COXs) are involved in the biosynthesis of PGs from arachidonic acid. Two isoforms of the enzyme have been described. COX-1 is constitutively expressed in most cells, whereas COX-2 is a readily inducible form arising in response to inflammatory stimuli.2 Conventional nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and aspirin, are nonspecific inhibitors of both COX-1 and COX-2.3 The nonspecific inhibitors, however, exhibit gastrointestinal side effects, including the formation of gastric and duodenal lesions,4 whereas selective COX-2 inhibitors reduce the manifestations of inflammation without development of significant gastrointestinal side effects.5

COX-1 and COX-2 convert arachidonic acid into the unstable endoperoxide intermediate PGH2. This rate-limiting step in PG synthesis provides the substrate for cell-specific isomerases and synthases that generate stable PGs.6 In inflammatory responses, COX-2 is induced, leading to increased synthesis of PGE2 and PGI2 from monocytes, macrophages, and vascular endothelium.7,8

Although COX-2 is regarded as an inducible enzyme, there is evidence that COX-2 is expressed constitutively in many tissues. The recent observation that COX-2 inhibition suppressed prostacyclin biosynthesis in healthy volunteers when administered over the dose range used in patients with osteoarthritis and rheumatoid arthritis indicates that constitutive COX-2 is a source of PG12 biosynthesis in humans.9 Crofford et al10 described 4 patients with connective tissue diseases in whom celecoxib was associated temporally with adverse vascular events. The patients exhibited increased levels of urinary PG12 metabolites, suggesting that selective COX-2 inhibition shifted the hemostatic balance toward a prothrombotic state. In hypertensive rats, selective COX-2 inhibition resulted in a significant increase in blood pressure and promoted leukocyte adherence to the vascular endothelium.11 Endothelial PG12 biosynthesis may counter the excessive activation of platelets in patients with diseases that predispose to thrombosis. Thus, inhibition of COX-2 and the
reduction in PGI2 synthesis may increase the risk for acute vascular events. Like conventional nonselective NSAIDs, selective COX-2 inhibitors also impair renal function.12,13

The goals of the present study were to investigate the antithrombotic efficacy of aspirin-induced COX-1 inhibition in the absence and presence of celecoxib and to determine whether the vasodilator responses to arachidonic acid in the coronary vascular bed were dependent on COX-2-derived prostacyclin. The results suggest that the antithrombotic actions of aspirin depend on maintaining endothelial COX-2-derived biosynthesis of prostacyclin. Furthermore, coronary artery vasodilator activity derived from the metabolism of arachidonic acid is diminished in the presence of COX-2 inhibition.

Methods

Guidelines for the Use and Care of Experimental Animals

Procedures used were in accordance with the guidelines of the University of Michigan Committee on the Use and Care of Animals and conform to the standards in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23). The University of Michigan Unit for Laboratory Animal Medicine provided veterinary care.

Model of Coronary Artery Thrombosis

The study group consisted of 65 purpose-bred dogs, 9 to 12 kg, anesthetized with sodium pentobarbital (30 mg/kg IV) and prepared for left circumflex coronary artery (LCx) thrombosis as previously described.14 After a flow probe and stenosis had been placed on the LCx, an intracoronary electrode was inserted through the LCx arterial wall, with the uninsulated portion positioned against the endothelial surface. An anodal current of 150 μA was applied to the endothelial surface to initiate the experiment. See Romson et al14 for details concerning the method and ultimate composition of the resulting occlusive thrombus.

Experimental Protocol

In the coronary artery thrombosis studies, animals were randomized into 5 groups. Figure 1 illustrates the dosing protocol for each group. In groups 3 and 5 (Figure 1), platelets remained irreversibly inhibited by aspirin throughout the endothelial recovery period, because the nonnucleated platelet lacks the capacity to synthesize new COX-1 protein. In contrast, COX-2 in the vascular endothelium is resynthe-

Figure 1. Protocol used to investigate effects of celecoxib and aspirin in model of coronary artery thrombosis. Dogs were randomized to receive placebo or drug as shown. Timing of hemato-

Hematological Parameters

Ex vivo platelet aggregation was assessed as previously described with arachidonic acid (0.65 mmol/L) and ADP (20 μmol/L) for induction of platelet aggregation.15 Tongue template bleeding times were determined with a Surgicut device (International Technidyne Corp). The lesion was blotted with filter paper every 20 seconds until the transfer of blood to the filter paper ceased.

Assessment of Coronary Artery Vasomotor Reactivity

Anesthetized dogs were instrumented for continuous measurement of arterial blood pressure and heart rate to monitor anesthesia and general condition of the animal preparation. An intracoronary infusion cannula consisting of a 5-mm distal section of a 25-gauge hypodermic needle fitted to a 10-cm length of polyethylene tubing (PE-90) (total volume 75 μL) was inserted through the wall of the LCx distal to the Doppler flow probe. The vasodilator responses to arachidonic acid or acetylcholine were recorded with a polygraph recorder interfaced with a PC computer and data acquisition software (Po-Ne-Mah, Gould Instrument Systems). Arachidonic acid or acetylcholine responses were determined before and after drug administration as described in the Results. Intracoronary infusions consisted of 90-μL drug injections followed by 200 μL 0.9% sodium chloride solution. Responses were normalized to vehicle injections of 290 μL. Ex vivo platelet activity was assessed before and after drug administration.

Drugs and Reagents

Celecoxib capsules were purchased from the hospital pharmacy. The capsule contents were weighed individually for oral administration or dissolved in 1% sodium bicarbonate (pH 12.5) immediately before intravenous injection. All remaining reagents were dissolved in saline. Furegrelate was purchased from Cayman Chemical Co. All other reagents were purchased from Sigma Chemical Co.

Statistics

Time to occlusive thrombus formation and tongue template bleeding times were compared by a 1-way ANOVA followed by Student-Newman-Keuls test. Ex vivo platelet aggregation values were compared by paired t tests. Coronary artery vasomotor responses were calculated as area under the blood flow response curve for 2 minutes after injection of arachidonic acid or acetylcholine and expressed as a change in volume blood flow (mL). The results were compared by a paired t test in the intravenous administration studies and a 1-way ANOVA in the groups receiving oral drug administration. All results were considered significant at a level of P<0.05. All data are expressed as mean±SEM.

Results

Effect of Celecoxib on Arterial Wall Injury and Occlusive Thrombus Formation

Time to occlusive thrombus formation was assessed in 30 dogs randomized into 5 groups as outlined in the Methods section (see Figure 1). Electrolytic injury of the LCx resulted in cyclic flow reductions that progressed to form an occlusive thrombus and sustained cessation of blood flow. The mean time for control animals to develop an occlusive arterial

sized during the endothelial recovery period. Throughout the report, we refer to high-dose aspirin (HDA) to indicate COX-1 and COX-2 inhibition in which the last dose of oral aspirin was administered 2 hours before the study was begun. In contrast, the group classified as HDA with endothelial recovery (HDA-ER) received the last oral dose of aspirin 17 hours before the start of the experimental protocol. The 17-hour aspirin-free period allowed time for the endothelium to recover its COX-2 activity through new protein synthesis while the platelet COX-1 activity remained inhibited.

Figure 1. Occlusive Thrombus Formation

Time to occlusive thrombus formation and tongue template bleeding times were compared by a 1-way ANOVA in the groups receiving oral drug administra-

**Statistics**

**Results**

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thrombus was 93±21.8 minutes (Figure 2). Oral administration of celecoxib (COX-2 inhibition) (2 mg/kg PO) or HDA (COX-1 and COX-2 inhibition) (4.6 mg/kg PO) did not produce significant changes in the time to occlusive arterial thrombus formation (celecoxib 77.7±7.2 minutes, HDA 72.0±18.5 minutes). The same dose of aspirin (4.6 mg/kg PO) followed by a 17-hour aspirin-free period to allow for endothelial recovery of COX-2 activity (HDA-ER) resulted in a significant increase in the time to occlusive thrombus formation (257.0±41.6 minutes). When oral celecoxib was administered (2 mg/kg PO) to animals in the HDA-ER group, the observed increase in time to occlusion was negated (80.7±20.6 minutes) (see Figure 2). The results suggest that the vasorelaxant and antiaggregatory actions of COX-2–derived prostacyclin may have an important physiological role and that selective inhibition of prostacyclin synthesis alters the antiplatelet benefit produced by HDA-ER.

Hematological Measurements in Thrombosis Studies
Ex vivo platelet aggregation in response to arachidonic acid was determined before and after administration of drug in each group described in Figure 2. Baseline percent platelet aggregation values ranged between 72.0% and 80.5% (Figure 3). Celecoxib alone (2 mg/kg PO) did not inhibit arachidonic acid–induced platelet aggregation, indicating its selectivity for COX-2. In groups 3 through 5, aspirin produced a significant inhibition of arachidonic acid–induced platelet aggregation characteristic of COX-1 inhibition (Figure 3). It should be noted that ex vivo arachidonic acid–induced platelet aggregation was inhibited in those animals in which aspirin had been withdrawn over the previous 17 hours. Ex vivo platelet aggregation in response to ADP was determined before and after drug administration in each group described in Figure 3. Neither aspirin nor celecoxib altered ADP-induced platelet aggregation. Despite being exposed to aspirin, platelets were able to respond ex vivo to ADP while not responding to arachidonic acid (data not shown).

The effects of celecoxib and aspirin on tongue bleeding time are summarized in Figure 4. Celecoxib alone did not affect bleeding time (2.1±0.2 minutes); however, animals treated with HDA and HDA-ER exhibited significant increases in tongue bleeding time (HDA 3.5±0.5 minutes, HDA-ER 4.2±0.4 minutes). Celecoxib-induced inhibition of COX-2 and reduction in endothelium-derived prostacyclin biosynthesis reversed the increase in bleeding time exhibited by animals treated with HDA-ER (2.9±0.2 minutes) (Figure 4). The observations suggest that the antiaggregatory and vasorelaxant properties of prostacyclin regulate platelet–vessel wall interactions and modulate in vivo platelet reactivity.

Effect of Celecoxib on Coronary Artery Vasomotor Responses
In vivo vasodilator responses to intracoronary administration of arachidonic acid or acetylcholine in the absence and presence of COX-2 inhibition were assessed. Figure 5 is a representative recording from 1 experiment. Before the administration of celecoxib, the intracoronary administration of 30 and 100 μg of arachidonic acid or 100 and 300 ng of acetylcholine increased coronary blood flow. In the same animal, 60 minutes after intravenous administration of 3.0 mg/kg of celecoxib, the vasodilator responses to 30 and 100 μg of arachidonic acid were decreased, whereas the responses to acetylcholine remained unchanged. Time controls were
performed to evaluate coronary responses over the course of an experiment, and no significant changes in the vasodilator responses to arachidonic acid or acetylcholine were observed (data not shown). The effect of sodium bicarbonate (pH 12.5), the vehicle for celecoxib, also was assessed and found to be without effect on coronary artery vasomotor tone (data not shown).

To quantify the vasodilator responses illustrated in Figure 5, the areas under the respective blood flow responses after the intracoronary administration of arachidonic acid or acetylcholine were calculated and converted to change in volume flow (mL). The method accounts for peak flow as well as for the duration of the vasodilator response. The dose-response relationship to arachidonic acid in the absence and presence of intravenous celecoxib (3 mg/kg) is shown in Figure 6a. Celecoxib significantly reduced the vasodilator responses to 30 and 100 μg of arachidonic acid, as determined by the change in volume flow. In contrast, the vasodilator effect of acetylcholine was unaltered after the administration of celecoxib.

In separate experiments, celecoxib was administered orally at a dose of 5.7 mg/kg. The oral dosing regimen resulted in a significant reduction of the vasodilator response to 30 and 100 μg arachidonic acid (Figure 6b).

**Effect of Aspirin on Coronary Artery Vasomotor Responses**

Vasodilator responses to arachidonic acid or acetylcholine were determined in each group receiving aspirin as described in Figure 1. In animals treated with HDA, vascular responses to arachidonic acid were reduced significantly compared with controls (Figure 7). In animals treated with HDA-ER, vasodilator responses to arachidonic acid were restored to values similar to control. When celecoxib was administered to animals treated with HDA-ER, vasodilator responses were reduced significantly (Figure 7). The data indicate that HDA inhibits vascular endothelial COX-2 in addition to platelet COX-1 and that HDA-ER permits regeneration of the endothelial COX-2.

**Effect of the Thromboxane Synthase Inhibitor Furegrelate on Coronary Artery Vasomotor Responses**

To determine whether or not the decreased vasodilator response to arachidonic acid observed in the presence of celecoxib was due to decreased prostacyclin (COX-2) or to increased thromboxane A2 (TxA2) production (thromboxane synthase), a TxA2 synthesis inhibitor, furegrelate, was administered. Furegrelate (3 mg/kg IV) did not alter the suppressed vasodilator responses mediated by celecoxib (Figure 8). In addition, furegrelate alone did not produce a change in the coronary artery vasomotor responses to arachidonic acid compared with control. The results demonstrate that the inhibition of arachidonic acid–induced vasodilation by celecoxib occurs because of decreased COX-2–derived prostacyclin production, rather than increased TxA2 synthesis.

To determine that furegrelate was effective at the dose administered, ex vivo platelet aggregation was assessed before and after administration of the TxA2 synthase inhibitor. Ex vivo platelet aggregation in response to arachidonic
In the present study, celecoxib was shown to alter inflammatory prostaglandins (PGs) produced via an induction of COX-2, whereas physiological selective COX-2 inhibitors was that inflammatory PGs are which also serves to inhibit platelet aggregation and as a part, from the capacity of endothelial cells to synthesize PGI₂, which also serves to inhibit platelet aggregation and as a mediator of inflammation.16,17 COX-2 is expressed at very low levels in most normal tissues but is induced rapidly in the presence of inflammation.18

The vascular endothelium is a source of vasoactive substances that function in modulating vascular tone, derived, in part, from the capacity of endothelial cells to synthesize PGI₂, which also serves to inhibit platelet aggregation and as a mediator of inflammation. The initial rationale for developing selective COX-2 inhibitors was that inflammatory PGs are produced via an induction of COX-2, whereas physiological PGs are generated from constitutive COX-1. It is now clear that COX-2 is constitutively expressed in the vascular endothelium.19 In the present study, celecoxib was shown to alter the protective hemostatic effects of aspirin by decreasing time to occlusive thrombus formation and reversing the increase in the aspirin-induced bleeding time. Celecoxib also suppressed coronary artery vasodilator responses to arachidonic acid, indicating that prostacyclin synthesis by COX-2 in the vascular endothelium may have important physiological functions in canine coronary arteries.

HDA did not prolong time to occlusive thrombus formation, despite inhibiting ex vivo platelet reactivity. At a dose of 4.6 mg/kg, aspirin inhibits the activity of platelet COX-1 and endothelial COX-2. The decreased vasodilator responses to arachidonic acid (Figure 7) confirm that endothelial COX-2 activity is inhibited at the administered dose of aspirin. Our previous studies demonstrated15,20 that short-term administration of aspirin (eg, HDA) did not exhibit antithrombotic effects in the electrolytic injury model unless the dose was increased beyond that needed to inhibit ex vivo platelet aggregation in response to arachidonic acid (20 mg/kg). The present data are consistent with our earlier studies and suggest that the failure of HDA to afford protection may be due to concomitant inhibition of COX-2 and suppression of endothelial PGI₂ synthesis.

HDA-ER increased time to thrombotic occlusion in response to endothelial injury and inhibited the ex vivo platelet response to arachidonic acid. The findings suggest that HDA-ER does not interfere with endothelium-related synthesis of PGI₂ (COX-2), while effectively inhibiting platelet COX-1 and preventing the synthesis of platelet-derived Tx₃. The vasodilator responses during HDA-ER indicate that endothelial COX-2 activity is restored and vasodilator responses to arachidonic acid are similar to those observed in the control group. Thus, the HDA-ER dosing regimen mimics the effects observed after treatment with low-dose aspirin, which inhibits platelet COX-1 while sparing endothelial COX-2. Our observation that celecoxib negates the increase in time to occlusion induced by HDA-ER suggests that COX-2–derived prostacyclin facilitates the antithrombotic effect observed with aspirin. When platelet COX-1 inhibition by aspirin is combined with the vasodilator and antiaggregatory actions of endogenous endothelium-derived prostacyclin biosynthesis, the net result is an antithrombotic response. In the absence of COX-2–derived prostacyclin, platelet COX-1 inhibition may be insufficient to prolong time to occlusive thrombus formation, because other platelet-activating mechanisms, such as ADP, serotonin, collagen, and thrombin, remain fully functional. Thus, the inhibition of endothelium-derived prostacyclin biosynthesis in the presence of aspirin shifts the hemostatic balance toward that of a prothrombotic state, especially in the presence of vascular wall injury. Because COX-2 is induced locally at the site of atherosclerotic lesions,21 the physiological functions of prostacyclin may be heightened in patients with coronary artery disease as well as those with diseases that predispose to occlusive vascular events.10

Our data indicate that COX-2 is involved in prolonging the time to occlusion mediated by HDA-ER. Treatment with celecoxib alone, however, did not influence the time to occlusive thrombus formation in these normal dogs. Previous studies in mice deficient in the receptor for prostacyclin suggest that the latter may serve to decrease the incidence of thrombosis.22 Ex vivo platelet reactivity was unaffected by celecoxib, consistent with its selectivity for COX-2. It is possible that during COX-1 inhibition with HDA-ER, an excess of arachidonic acid is made available for metabolism by both the COX-2 and lipoxygenase pathways. The phenomenon of an endoperoxide shunt after treatment with HDA-ER may generate a heightened antithrombotic role for COX-2–derived prostacyclin that is not present in the absence of HDA-ER. The endoperoxide shunt has been described in vitro when bovine coronary artery tissue is incubated with human platelets23 and more recently in human umbilical vein endothelial cells coincubated with platelets.24 Additional studies are needed, however, to demonstrate the presence of an in vivo endoperoxide shunt.

To demonstrate a functional effect of endothelial COX-2 in the coronary circulation, vasodilator responses to intracoro-
nary infusions of arachidonic acid were assessed in the anesthetized dog before and after the administration of the selective COX-2 inhibitor celecoxib. Celecoxib suppressed the increase in coronary artery blood flow induced by the intracoronary administration of arachidonic acid. Under the same experimental conditions, the vasomotor responses to the endothelium-dependent vasodilator acetylcholine were not affected by the previous administration of celecoxib. The observations are interpreted as demonstrating a functional role for constitutive COX-2 in the coronary vasculature. Because HDA also suppressed the vasodilator responses, this is further support for a functional role for constitutive COX-2.

One limitation of the present study was the inability to assay for the PGI: metabolite 6-keto PGF\(_6\), in blood or urine. Furthermore, we cannot rule out other prostanoids as possible mediators of the vasodilator response. Because celecoxib effectively reduces the responses to arachidonic acid, we can conclude that the mediators of the vasodilator response are most likely products of COX-2. In the endothelium and blood platelets, arachidonic acid and PGH\(_2\) may act as substrates for COX-1 and TxA\(_2\) synthase, respectively. Therefore, we considered the possibility that an endoperoxide shunt could explain the celecoxib-induced suppression of the coronary vasodilator response to arachidonic acid. The results with furegrelate indicate that an endoperoxide shunt leading to the formation of TXA\(_2\) in the presence of COX-2 inhibition is unlikely to account for the decreased reactivity of the coronary artery responses to arachidonic acid.

The results of the present study indicate that endogenous endothelial COX-2-derived prostacyclin participates in the cardioprotective effects afforded by HDA-ER. This is demonstrated by the observation that celecoxib reversed the aspirin-induced inhibition of in vivo coronary thrombosis and tongue bleeding time. We also demonstrate that constitutive COX-2 expression in the vascular endothelium mediates the coronary vasodilator events in response to intracoronary arterial administration of arachidonic acid. The inhibition of COX-2 with a reduction in endogenous prostacyclin biosynthesis may therefore be associated with an increase in adverse clinical outcomes, especially in individuals predisposed to vasculopathy and thrombosis.\(^{10,25}\)

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