Selective COX-2 inhibition significantly decreased the prostaglandin lar reactivity atherosclerosis; cyclooxygenase; eicosanoids; prostaglandins; vascular sclerotic lesions.

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Atherosclerosis is characterized by chronic inflammation and enrichment of inflammatory cells, such as T lymphocytes and macrophages, in the vascular wall (16). These cells release proinflammatory cytokines and regulate the production of other mediators such as eicosanoids [prostaglandins (PG), thromboxanes (TX), and leukotrienes] (2, 25). Because of the implications of the latter mediators in cardiovascular physiology and since their biosynthesis is severely altered in patients with atherosclerosis, eicosanoids may be important mediators of atherosclerosis (2, 21).

PGs are synthesized from arachidonic acid via cyclooxygenase (COX) activities. Two isoforms of the COX enzyme, which derive from two distinct genes, have been described where COX-1 is constitutively expressed, whereas the COX-2 expression is induced specifically under inflammatory conditions (14). An increase of the COX-2 activity is associated with many pathological situations such as arthritis, cancer, and in inflammatory conditions related to atherosclerosis (10, 25, 27).

The present study was to evaluate the role of COX-2 induced by an atherosclerotic process, in the local control of vascular tone. New Zealand White rabbits were fed 0.3% cholesterol and subjected to balloon injury of the abdominal aorta. After 2 wk, the aorta was removed and used for organ bath experiments and immunohistochemistry, and the prostaglandins released were measured using enzyme immunoassays. Hypercholesterolemia and vascular injury significantly increased the thickness of the intimal layer, which was associated with an induction of COX-2 immunoreactivity throughout the aortic wall. In these preparations, a significant decrease of the maximal contractions induced by norepinephrine was observed. The norepinephrine-induced contractions of atherosclerotic preparations were restored by the COX inhibitors DuP-697 (0.5 \( \mu \)mol/l) and indomethacin (1.7 \( \mu \)mol/l), to similar contractions as was observed in normal preparations derived from healthy rabbits. Norepinephrine stimulation of the abdominal aorta was accompanied by increased levels of prostaglandin I\(_2\) but not of prostaglandin E\(_2\), prostaglandin D\(_2\), or thromboxane A\(_2\) in atherosclerotic compared with normal aorta. Selective COX-2 inhibition significantly decreased the prostaglandin I\(_2\) release from atherosclerotic aorta but had no effect on the prostaglandin release from aortic preparations derived from normal rabbits. These observations suggest that the local induction of COX-2 during atherosclerosis decreased the sensitivity to norepinephrine and that COX-2 inhibitors may increase vascular reactivity at sites of atherosclerotic lesions.

Altered reactivity to norepinephrine through COX-2 induction by vascular injury in hypercholesterolemic rabbits

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Submitted 3 February 2009; accepted in final form 10 September 2009

Foudi N, Norel X, Rienzo M, Louedec L, Brink C, Michel JB, Bäck M. Altered reactivity to norepinephrine through COX-2 induction by vascular injury in hypercholesterolemic rabbits. Am J Physiol Heart Circ Physiol 297: H1882–H1888, 2009. First published September 25, 2009; doi:10.1152/ajpheart.00092.2009.—Although long-term use of cyclooxygenase (COX)-2 inhibitors may be associated with increased cardiovascular risk, their effects on vascular reactivity in atherosclerosis has remained largely unexplored. The aim of the present study was to evaluate the role of COX-2 induced by an atherosclerotic process, in the local control of vascular tone. New Zealand White rabbits were fed 0.3% cholesterol and subjected to balloon injury of the abdominal aorta. After 2 wk, the aorta was removed and used for organ bath experiments and immunohistochemistry, and the prostaglandins released were measured using enzyme immunoassays. Hypercholesterolemia and vascular injury significantly increased the thickness of the intimal layer, which was associated with an induction of COX-2 immunoreactivity throughout the aortic wall. In these preparations, a significant decrease of the maximal contractions induced by norepinephrine was observed. The norepinephrine-induced contractions of atherosclerotic preparations were restored by the COX inhibitors DuP-697 (0.5 \( \mu \)mol/l) and indomethacin (1.7 \( \mu \)mol/l), to similar contractions as was observed in normal preparations derived from healthy rabbits. Norepinephrine stimulation of the abdominal aorta was accompanied by increased levels of prostaglandin I\(_2\) but not of prostaglandin E\(_2\), prostaglandin D\(_2\), or thromboxane A\(_2\) in atherosclerotic compared with normal aorta. Selective COX-2 inhibition significantly decreased the prostaglandin I\(_2\) release from atherosclerotic aorta but had no effect on the prostaglandin release from aortic preparations derived from normal rabbits. These observations suggest that the local induction of COX-2 during atherosclerosis decreased the sensitivity to norepinephrine and that COX-2 inhibitors may increase vascular reactivity at sites of atherosclerotic lesions.

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PGs are synthesized from arachidonic acid via cyclooxygenase (COX) activities. Two isoforms of the COX enzyme, which derive from two distinct genes, have been described where COX-1 is constitutively expressed, whereas the COX-2 expression is induced specifically under inflammatory conditions (14). An increase of the COX-2 activity is associated with many pathological situations such as arthritis, cancer, and in inflammatory conditions related to atherosclerosis (10, 25, 27). PGs play a major role in the control of the vascular tone. In isolated vessels, PGD\(_2\) and PGI\(_2\) cause vasodilatation, PGF\(_{2 \alpha}\) and TXA\(_2\) induce vasoconstriction, while PGE\(_2\) may produce both effects (12, 21). Generally, normal vascular walls produce significant levels of PGI\(_2\) compared with the other prostanoids. However, under certain inflammatory conditions, the profile of PG secretion from the vascular wall may change (3, 13, 28). These effects have, for example, been observed in cells submitted to proinflammatory stimuli such as lipopolysaccharide and interleukin-1\( \beta \) (9, 11) in association with the induction of COX-2.

Then, theoretically, COX-2 blockade could be beneficial in atherothrombosis. In fact, the COX-2 inhibition decreases the inflammatory response and intimal hyperplasia in a rabbit balloon injury model (30). However, the inhibition of COX-2 by coxibs such as rofecoxib has been associated with cardiovascular events (7, 8, 19, 24). The latter effects have been suggested to be due to an imbalance between PGI\(_2\) and TXA\(_2\) synthesis, which act as anti- and prothrombotic mediators, respectively (15). COX-2 inhibition does not suppress COX-1-derived TXA\(_2\) production in platelets, unlike nonselective nonsteroidal anti-inflammatory drugs, thereby tipping the TXA\(_2\)/PGI\(_2\) balance toward a prothrombotic state.

There are few in vitro studies concerning a physiological role of COX-2 in the cardiovascular system (13, 23). In human vessels, COX-2 induction by proinflammatory stimulation ex vivo alters the response to norepinephrine (NE). However, although increased COX-2 expression has been shown to alter vascular reactivity in animal models of hypertension (1), the association between the expression of COX isoforms and their respective role in the control of the vascular reactivity has not been explored in atherosclerosis. Therefore, the aim of this study was to examine the role of COX-2 in the regulation of vascular reactivity induced by NE in a rabbit model of atherosclerosis on isolated vessels in the absence of blood cells and platelets. Our hypothesis was that the local induction of COX-2 could directly modify the vascular tone at sites of atherosclerotic lesions.
MATERIALS AND METHODS

Animal protocols. The study was approved by the institutional Animal Care and Use Committee. Five male New Zealand White rabbits (3.5–4.0 kg; CPA, Olivet, France) received a diet containing 0.3% cholesterol (SAFE, Augy, France) starting 3 wk before the vascular injury and continued until death. The balloon dilatation was performed as previously described (18). Briefly, premedication with intramuscular 1% acepromazine (Vetranquil) was followed by intravenous anesthesia with 0.1% pentobarbital sodium. Local anesthesia with xylocaine was also administered subcutaneously at the site of the femoral incision. Heparin (500 IU iv) was administered once the arterial access was obtained. A 5-French introducer was installed in the femoral artery through which a 4-mm-diameter/40-mm-length angioplasty balloon was installed. The balloon was inflated three times (30 s, 10 ATM) in segments of the abdominal aorta (AA) between the renal arteries and the iliac bifurcation under fluoroscopic guidance using a Philips BV Endura fluoroscope. After repair of the femoral skin wound, rabbits were left to recover from anesthesia under surveillance in a temperature-controlled room. Four more rabbits, receiving ordinary chow without cholesterol and not being subjected to the angioplasty protocol, were also included in the study.

Rabbit tissue preparations. The rabbits were killed by an overdose of pentobarbital sodium 14 days after balloon injury. AA and thoracic aorta (TA) were harvested, flushed with saline, and cleaned of adipose tissue, followed by either fixation in 4% paraformaldehyde or organ bath experiments. The kidneys were removed and placed immediately in 4% paraformaldehyde for immunohistochemistry experiments.

Morphometric and histological analysis. Arterial preparations were embedded in paraffin after paraformaldehyde fixation and used for analysis of intima, media, and adventitia surfaces. Following deparaffinization and rehydration, transversal slices (5 μm) of AA were stained with hematoxylin, Masson’s trichrome, or Orceine. Intima and media area were measured using Histolab Software.

Immunohistochemistry. Other sections of AA and kidney were used for immunohistochemical studies. Endogenous peroxidases were blocked by hydrogen peroxide (Sigma), and antigen retrieval was performed in antigen unmasking solution (Vector). The polyclonal anti-human COX-1 and COX-2 antibodies (Santa Cruz) were used at 1:50 dilution at 4°C overnight. Biotinylated anti-goat was the secondary antibody, and peroxidase Vectastain Elite ABC kits were used for detection. Negative controls were performed in absence of antibody. The staining was quantified using Histolab Software as previously described (18), and the results were expressed as percent of either intima, media, or adventitia area.

Organ bath and isometric measurements. Three to 4 AA and TA preparations from each rabbit, cut as rings of 3 mm of width, were set up in organ baths as previously described (13). An initial concentration-response curve was performed with NE (Sigma) added in a...
cumulative fashion to the baths (1 nmol/l-100 μmol/l). When the maximal effect was obtained, the preparations were washed with fresh Tyrode’s solution until they returned to the resting tone. Subsequently, these vascular preparations were incubated (30 min) in either Tyrode’s solution or Tyrode’s solution containing one of the following COX inhibitors: Indomethacin (1.7 μmol/l; Sigma) or the selective COX-2 inhibitor DuP-697 (0.5 μmol/l; Cayman) before a second concentration-response curve of NE (1 nmol/l-100 μmol/l).

Prostanoid measurements. Tyrode’s solution was collected after the incubation period (30 min) and at the end of the concentration-response induced by NE. The concentrations of PGE2, PGD2, TXB2, a stable metabolite of TXA2 and 2,3-dinor-6-keto-PGF1α, a stable metabolite of PGI2, were measured using an enzyme immunoassay kit (Cayman) according to the manufacturer’s instructions. The results are expressed as picograms per milligram of wet weight tissue.

Data analysis. Acquisition and processing of the physiological data were performed with the IOX software (EMKA) and expressed in grams. The pEC50 values were calculated as the negative log of the half-maximum effective concentration (EC50 values). All data are presented as means ± SE derived from n rabbits. The concentration-response curves were analyzed by two-way ANOVA and Student-Newman-Keul’s post hoc test. The pEC50, Emax, immunohistochemistry quantifications, and PG measurements were analyzed by paired Student’s t-test or Student’s t-test. A P value < 0.05 was considered statistically significant.

Fig. 2. Representative immunostainings (A) and quantifications (B) for cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in abdominal aortic sections obtained from normal (n = 3) and atherosclerotic (Cholesterol + Ballooning, n = 5) rabbits. Magnification X10. *Data significantly different vs. normal rabbits (P < 0.05; Student’s t-test). Negative control was performed without antibody.

Fig. 3. Contraction of rabbit abdominal aortic preparations (36 rings) induced by norepinephrine (NE; first concentration-response curve) in normal (n = 4) or atherosclerotic (Cholesterol + Ballooning, n = 5) rabbits. These data were significantly different; P < 0.05, ANOVA.
RESULTS

Plasma cholesterol. The 0.3% cholesterol regimen significantly increased plasma cholesterol. The plasma levels of cholesterol at the time of death were $339 \pm 97$ mg/dl ($n = 4$) and $18 \pm 3.3$ mg/dl ($n = 3$) for cholesterol-fed and chow-fed rabbits, respectively.

Histological analysis. The histological analysis revealed a significant increase of the intimal layer of AA obtained from rabbits subjected to vascular injury and fed the 0.3% cholesterol regimen (Fig. 1). The intima-to-media ratios were 0.04 and 0.37 for atherosclerotic and normal rabbits, respectively. This effect was accompanied by smooth muscle cell proliferation and an increase of elastic lamellae quantity as shown by Masson’s trichrome and Orceine colorations, respectively.

COX expression in rabbit AA. Data derived from the immunohistochemical experiments (Fig. 2) demonstrated that the COX-1 isoform was similarly but only slightly expressed in

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal ($n = 4$)</th>
<th>Cholesterol + Ballooning ($n = 5$)</th>
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<tbody>
<tr>
<td></td>
<td>pEC$_{50}$</td>
<td>$E_{\text{max}, g}$</td>
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<tr>
<td>Without inhibitor</td>
<td>6.15±0.03</td>
<td>9.05±2.68</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>5.80±0.16</td>
<td>9.17±2.73</td>
</tr>
<tr>
<td>DuP-697</td>
<td>6.06±0.07</td>
<td>9.33±1.51</td>
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Values are means ± SE; $n$, no. of rabbits. pEC$_{50}$ and $E_{\text{max}, g}$ for the second concentration-response curve performed with norepinephrine in rabbit abdominal aortic preparations in the absence or presence of cyclooxygenase inhibitors (1.7 μmol/l indomethacin and 1 μmol/l DuP-697; 30 min). $P < 0.05$, Student’s paired $t$-test vs. normal rabbits (*) and Student’s $t$-test vs. without inhibitor (†).
either normal or atherosclerotic AA preparations. This expression was mainly localized in the adventitia. In contrast to AA, the COX-1 isoform was markedly expressed in kidney preparations using the same antibody ($n = 3$, data not shown). In contrast, the COX-2 isoform was predominantly expressed in AA preparations obtained from atherosclerotic rabbits and was localized in all three layers of the vascular wall.

**Vasoconstriction induced by NE.** The first dose-response curve and the maximal contraction induced by NE on AA were significantly reduced in atherosclerotic rabbits (Fig. 3). The pEC$_{50}$ values were not significantly different ($6.13 \pm 0.06$ and $6.02 \pm 0.19$ for normal and atherosclerotic rabbits, respectively).

**Effects of COX inhibitors on NE-induced vasoconstriction.** To evaluate the involvement of COX activities in the control of the vascular tone, the second dose-response curve to NE was performed in the absence or presence of COX inhibitors. The effects of COX inhibitors on pEC$_{50}$ and E$_{max}$ are shown in Table 1. Again, no difference in pEC$_{50}$ was observed between normal and atherosclerotic rabbits. DuP-697 and indomethacin increased the contractions and maximal effects induced by NE only in AA preparations obtained from atherosclerotic rabbits (Table 1 and Fig. 4B). In contrast, the COX inhibitors did not significantly alter the NE-induced contractions of the TA derived from either normal or hypercholesterolemic rabbits in either group using TA preparations (Fig. 4, C and D).

**Effect of COX inhibitors on prostanoid release from AA preparations.** In absence of COX inhibitors, the release of 2,3-dinor-6-keto-PGF$_{1 \alpha}$ by AA in the organ bath medium was increased in atherosclerotic compared with normal rabbits, whereas PGD$_2$, TxB$_2$, and PGE$_2$ productions were not changed (Fig. 5). The 2,3-dinor-6-keto-PGF$_{1 \alpha}$ production was $\sim$100- to 1,000-fold greater compared with the other prostanoids measured (Fig. 5).

The 2,3-dinor-6-keto-PGF$_{1 \alpha}$ released in organ bath medium containing AA from atherosclerotic rabbits was significantly decreased in the presence of COX inhibitors after NE stimulation (Fig. 5), an effect not observed in AA from normal rabbits, whereas PGD$_2$, TxB$_2$, and PGE$_2$ release were unchanged after COX inhibition in both types of AA preparations. The NE stimulation caused a significant increase of 2,3-dinor-6-keto-PGF$_{1 \alpha}$ production in organ bath medium containing AA from atherosclerotic but not from normal rabbits, an effect not observed with PGD$_2$, TxB$_2$, and PGE$_2$ release (Fig. 5).
DISCUSSION

The results of the present study point to an important vasomotor effect of COX-2 induction induced at the site of the atherosclerotic lesion, leading to a local release of vasorelaxant PGI2. These findings support previous in vitro work in isolated human vessels in which COX-2 inhibitors increased the vascular tone of isolated arteries submitted to inflammatory conditions (13). In the present rabbit model, cholesterol and balloon injury induced an atherosclerotic morphology of the AA and an increase of the intima thickness, which was accompanied by a substantial induction of COX-2.

The minor expression of COX-1 in the AA corroborates with previous work in rabbit vessels, such as femoral (28), intralobar pulmonary (20), carotid (17), and small mesenteric (32) arteries. The present study extends those previous studies by showing a similar COX-1 expression in the aorta of normal and atherosclerotic AA, which was mainly localized in the adventitia. Although the COX-2 was not expressed in AA under normal conditions, a marked COX-2 immunostaining in AA was observed in atherosclerotic AA and localized in the entire vascular wall. The latter results are in contrast to a previous study showing an absence of COX-2 induction in AA from cholesterol-fed rabbits without vascular injury (31). These findings suggest that the vascular injury was more potent to induce COX-2 expression than hypercholesterolemia in the present model. The latter notion is supported by the COX-2 induction after femoral vascular injury in rabbits (30). Interestingly, the aortic induction of COX-2 expression in the present study is similar to what has been reported for human atherosclerotic lesions (10, 25, 27).

In the present study, the induction of COX-2 protein in the aorta derived from atherosclerotic rabbits was accompanied by a decreased vascular reactivity to NE. Furthermore, whereas both the unselective COX inhibitor indomethacin and the selective COX-2 inhibitor DuP-697 restored the NE-induced contractions in AA derived from atherosclerotic rabbits, those inhibitors were without effect in normal vessels. In contrast, the TA, which in the present study was not subjected to vascular injury, exhibited no significant alterations of NE reactivity by COX inhibitors in preparations derived either control or hypercholesterolemic rabbits. Taken together, the decreased vascular tone observed in the AA in vivo studies using human, porcine, and rat vessels in which the decreased reactivity to NE, KCl, or U-46619, was related to COX-2 induction (13, 23, 29).

In the present study, the release of PGI2 was >100-fold greater than the release of TxA2, PGD2, and PGE2 in both normal and atherosclerotic rabbits. Although the latter observations confirm previous results in rabbit aorta (5, 6, 26, 31), studies on the effects of atherosclerosis on prostaglandin release have generated contradictory results. For example, hypercholesterolemic rabbits have been reported to exhibit both an increased (6) and a decreased (5, 26) PGI2 release from the aorta, as measured by 6-keto-PGF1α release. Furthermore, no significant difference in either PGE2 or 6-keto-PGF1α release after arachidonic acid stimulation of aortas derived from either normal or hyperlipidemic rabbits has been reported (31). There are, however, two important differences between the present and the previous findings, namely the aortic induction of COX-2 by balloon injury in our model and the measurements of PGI2 release by quantification of 2,3-dinor-6-keto-PGF1α, a more stable metabolite compared with 6-keto-PGF1α (4). Furthermore, the agonist used for vascular stimulation may influence the degree of COX activation. In the present study, whereas unstimulated samples did not exhibit significant differences in terms of prostaglandin release, NE increased the release of PGI2 only in aorta from atherosclerotic rabbits. Similar activation of COX after NE stimulation has also been observed in previous studies of systemic and pulmonary vessels (13, 22).

The massive COX-2 staining observed throughout the vascular wall after balloon dilatation in the present study is in contrast to the scarce COX-2 staining limited to macrophages in a study of rabbits with comparable cholesterol values but without vascular injury (31). In addition, the latter study did not detect aortic COX-2 protein by Western blot, whereas COX-1 expression was present. Finally, the PGI2 and PGE2 release induced by cholesterol diet alone was not inhibited by selective COX-2 inhibition (31), which is in contrast to the reduction of PGI2 release observed after pretreatment with DuP-697 in the present study. Taken together, these findings suggest that COX-2 induction is a prerequisite for an increased local PGI2 production within atherosclerotic lesions.

In AA derived from normal rabbits, the release of PGI2 was significantly lower compared with atherosclerotic AA, and the COX-2 inhibitor DuP-697 did not significantly alter either the PGI2 release or the NE-induced contractions. These observations were in accordance with the immunohistochemical results of the present study, where COX-1 was only slightly expressed, and suggest a minor role of COX-1 activity in the control of the NE-induced vascular tone.

In summary, the present study demonstrates that the atherosclerotic morphology induced by hypercholesterolemia and vascular injury was associated with a substantial COX-2 induction, leading to an increase of PGI2 synthesis that exerted significant effects on NE-induced vascular tone. Those effects were reversed and the vascular tone restored after treatment with a COX-2 inhibitor. These results suggest that the cardiovascular risk of coxibs that emerged from clinical trials may in addition be associated with a local increase of the vascular reactivity at sites of atherosclerotic lesions. In conclusion, inhibition of COX-2 would not only alter the thrombotic homeostasis but also the vascular tone locally in atherosclerotic vessels through inhibition of PGI2 release.

GRANTS

This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM, France), Leducq Foundation, Académie Nationale de Médecine (France), Mairie de Paris (“Research in Paris”), The Swedish Heart and Lung Foundation, and The French-Swedish Foundation.

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