Mechanism for Covalent Binding of Rofecoxib to Elastin of Rat Aorta

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ABSTRACT

We have previously reported that oral administration of [14C]rofecoxib to rats resulted in the long retention of radioactivity by the aorta as a consequence of covalent binding to elastin. Treatment of rats with α-phenyl-α-propylbenzeneacetic acid 2-[diethylamino]-ethyl ester hydrochloride (SKF-525A), a cytochrome P450 inhibitor, significantly decreased the systemic exposure of unchanged rofecoxib in the dose range between 2 and 10 mg/kg. A covalent binding study of [14C]rofecoxib in vitro using rat aorta homogenate in the presence of d-penicillamine, hydralazine, β-aminopropionitrile, and sodium borohydride suggested that the aldehyde group of allysine in elastin was relevant to the covalent binding. In a model reaction using benzaldehyde, rofecoxib but not 5-hydroxyrofecoxib reacted with the aldehyde group of benzaldehyde in a manner of condensation reaction under a physiological pH condition. A histopathological examination using an electron microscope demonstrated that multiple oral administration of rofecoxib to rats caused marked degradation of the elastic fiber system of the aorta. These results suggested that rofecoxib as such is reactive in vivo, undergoing a condensation reaction with allysine, thereby preventing the formation of cross-linkages in elastin, i.e., desmosine and isodesmosine, and causing the degradation of the elastic fibers.

Rofecoxib [3-phenyl-4-[4-(methylsulfonyl)phenyl]-2-(5H)-furanone, VIOXX] is a potent and highly selective cyclooxygenase-2 (COX-2) inhibitor and had been widely used as a nonsteroidal anti-inflammatory drug (NSAID). In 2004, however, it was withdrawn from the market on the basis of the results of some clinical studies, indicating association of its use with an increased risk of adverse cardiovascular (CV) events, such as heart attack and stroke (Merck announces voluntary worldwide withdrawal of VIOXX: http://www.vioxx.com/vioxx/documents/english/vioxx_press_release.pdf, 2004).

In the last few years, it has been reported that other selective COX-2 inhibitors (e.g., etoricoxib, parecoxib, and valdecoxib) and nonselective NSAIDs (e.g., naproxen) may also have a potential for increased CV risk (Aldington et al., 2005; Nussmeier et al., 2005) (see “Use of Non-Steroidal Anti-Inflammatory Drugs Suspended in Large Alzheimer Disease Prevention Trial” in http://www.nih.gov/news/pr/dec2004/od-20.htm). However, rofecoxib differs in the following ways: 1) a significantly greater frequency and higher odds of CV events (Mamdani et al., 2004; Solomon et al., 2004a; Graham et al., 2005; Kimmel et al., 2005); 2) a shorter period and a lower dose (even at a clinical dose) leading to the incidence of CV events (Solomon et al., 2006); and 3) an earlier onset and a greater hypertensive effect correlating closely with CV risk (Brinker et al., 2004; Solomon et al., 2004b; Wolfe et al., 2004; Frey et al., 2005). Therefore, it is suggested that rofecoxib could have distinctive mechanisms or more toxic potential, leading to CV risks, compared with other selective COX-2 inhibitors or nonselective NSAIDs.

Regarding its mechanism, several hypotheses have been proposed so far. However, most of them are common to all selective COX-2 inhibitors or nonselective NSAIDs and not
specific to rofecoxib, e.g., prostacyclin/thromboxane A₂ imbalance in arteries (McAdam et al., 1999) and disruption of the production of prostaglandins, which play an important homeostatic role in the kidney (Pope et al., 1993; Johnson et al., 1994). On the other hand, Walter et al. (2004) proposed the pro-oxidant effect theory, whereby rofecoxib, which is a sulfone-type COX-2 inhibitor, promotes oxidative damage to low-density lipoprotein and phospholipids in vitro, and this action might lead to atherogenesis in vivo. This theory might be able to account for the high incidence of CV events with use of rofecoxib, but it has not yet been proven in vivo. Reddy and Corey (2005) reported that the lactone ring in rofecoxib is capable of undergoing spontaneous oxidation. Furthermore, they report that if one of the resulting metabolites, its maleic anhydride form, is generated transiently in vivo, even though this has not been detected at all, it could react with nucleophilic groups in the biomolecules, especially amino acids. Even with consideration of this possibility, however, it is difficult to explain why the radioactivity is retained only in a limited number of tissues, such as the aorta, ligament, and cartilage (Oitate et al., 2006).

In our previous study, we demonstrated that the radioactivity from [¹⁴C]rofecoxib was covalently bound to the arterial elastin of rats (Oitate et al., 2006). Elastin, a natural elastomer, is a key extracellular matrix protein, which provides CV tissues, e.g., arteries and heart valves, with tensile strength and elasticity, and maintains the tissue architecture (Vrhovski and Weiss, 1998). This physical property of elastin is due to covalent cross-linkage structures, such as desmosine and isodesmosine (see Fig 7A). These cross-linkages are preceded by selective lysine oxidation by the enzyme lysyl oxidase (LOX) (EC 1.4.3.13) to produce a reactive aldehyde, ω-aminoadipic-δ-semialdehyde (allysine), which can spontaneously react with neighboring aldehydes or ε-amino groups to form cross-linkages. It has been reported that an anti-rheumatic amino thiol, δ-penicillamine, reacts with the aldehyde group of allysine to form a thiazolidine-type complex (Pinnell et al., 1968; Deshmukh and Nimni, 1969; Howard-Lock et al., 1986). Chronic administration of this compound has also been known to cause deep lesions in the connective tissues, such as angiopathy and pseudoxanthoma elasticum in rats and humans (Hashimoto et al., 1981; Junker et al., 1982; Light et al., 1986). From this information, we hypothesized that rofecoxib and/or its metabolite(s) might also form a covalent adduct with allysine in elastin and cause damage to the elastin.

In the present study, we investigated the mechanism for covalent binding of rofecoxib to elastin using in vivo and in vitro approaches, especially focusing on the possibility that rofecoxib, but not 5-hydroxyrofecoxib, a metabolite of rofecoxib, can bind to the aldehyde group of allysine. Furthermore, histopathological changes in the aortic walls of rats were examined by transmission electron microscopy after multiple oral administration of rofecoxib.

Materials and Methods

Chemicals and Reagents

[¹⁴C]rofecoxib (17 mCi/mmol) was synthesized at GE Healthcare (Little Chalfont, Buckinghamshire, UK). The radiochemical purity was >99% in analysis by radiodetection-high performance liquid chromatography (HPLC). Nonradiolabeled rofecoxib, 5-hydroxyrofecoxib, and valdecoxib, which was used as an internal standard substance for analysis of rofecoxib and 5-hydroxyrofecoxib by liquid chromatography (LC)-tandem mass spectrometry (MS/MS), were synthesized at Sankyo Co., Ltd. (Tokyo, Japan). Polyethylene glycol 400 (PEG 400) was purchased from Wako Pure Chemicals (Osaka, Japan). ω-Penicillamine, hydralazine, β-aminopropionitrile (BAPN), sodium borohydride (NaBH₄), and benzaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Deuterated acetanilide (CD₂CN, 99.9% deuterium) and deuterium oxide (D₂O, 99.9% deuterium) were purchased from Isotec Inc. (Miamisburg, OH) and used to prepare the solvents for LC-nuclear magnetic resonance (NMR) analysis. All other reagents and solvents used were commercially available and were of extra pure, guaranteed or HPLC or LC/MS grade.

Dosing of Animals and Sample Collection

Male Sprague-Dawley rats (6 weeks of age) were obtained from Charles River Japan, Inc. (Yokohama, Japan) and were used after 1 week of acclimatization. The rats were housed in a temperature-controlled room with a 12-h light/dark cycle. Their body weights ranged from 210 to 230 g at the start of dosing. The rats were fasted overnight before the dosing. Water was available ad libitum throughout the study.

[¹⁴C]Rofecoxib (for radioactivity measurement in the aorta) or nonradiolabeled rofecoxib (for analysis of rofecoxib and 5-hydroxyrofecoxib in plasma) was dissolved in PEG 400 and administered p.o. to rats at a dose of 2, 5, or 10 mg/2 ml/kg (n = 3). SKF-525A (50 mg/kg, Funakoshi, Tokyo, Japan) in isotonic saline was injected i.p. into the rats at 0.5 h before the administration of [¹⁴C]rofecoxib (2 mg/kg) or nonradiolabeled rofecoxib (2 mg/kg). Blood samples were collected with heparinized syringes at 0.5, 1, 2, 4, 6, 8, 10, and 24 h after the dosing of nonradiolabeled rofecoxib under diethyl ether anesthesia, and plasma was subsequently obtained by centrifugation. At 24 h after administration of [¹⁴C]rofecoxib, the thoracic aortas were collected from the rats after they were euthanized by exsanguination under diethyl ether anesthesia.

For the histopathological study, rofecoxib was administered p.o. repeatedly to rats once daily for 4 weeks except on weekends (five times a week) at a dose of 10 mg/2 ml/kg (n = 6). As a control, the vehicle (PEG 400) was administered p.o. (2 ml/kg, n = 6). At 72 h after the final dosing, the rats were euthanized by exsanguination under diethyl ether anesthesia, and the thoracic aortas were collected.

Covalent Binding of [¹⁴C]Rofecoxib in Vitro to Rat Aorta Homogenate and Effect of Protein Modifiers

The thoracic aorta samples were obtained from untreated rats (n = 13). After removal of the adhering tissues, the aortic samples were pooled, weighed (~600 mg, wet weight), and homogenized in isotonic saline (5% w/v) using a motor-driven homogenizer. According to previous reports (Tang et al., 1983; Wilmarth and Froines, 1992; Ohta et al., 1998), the homogenates were pretreated with protein modifiers, δ-penicillamine (10 mM), hydralazine (10 mM), BAPN (10 mM), or NaBH₄ (20 mM) in potassium phosphate buffer (100 mM, pH 7.4) at 37°C for 0.5 h and then incubated with [¹⁴C]rofecoxib (100 μM) at 37°C for 2 h (n = 3). As a control, the homogenate was pretreated with buffer alone. For evaluation of nonspecific binding, the same treatments were performed at 4°C for 2 h (n = 3). After the incubation, 0.9 M trichloroacetic acid was added to the incubation mixture to precipitate the proteins. Then, the mixture was centrifuged at 4°C for 10 min. The supernatant was removed for radiodetection-HPLC analysis, and the precipitate was washed by resuspension and centrifugation successively with 0.8 M trichloroacetic acid, 80% (v/v) methanol, and 100% methanol. This process was repeated twice. The resulting precipitates were air-dried and subjected to radioactivity measurement. The net amount of covalent binding was calculated by subtracting the nonspecific binding from the total.
LOX activity in the presence of either of the protein modifiers or rofecoxib was measured using an Amplex Red fluorescence assay (Invitrogen, Carlsbad, CA) (Payne et al., 2005). The assay reaction mixture consisted of 55.6 mM sodium borate (pH 8.2), 1.33 mM urea, 55.6 mM Amplex Red, 0.11 U/ml horseradish peroxidase, and 11.1 mM 1,5-diaminopentane (cadaverine) substrate (final concentration: 100%). The experiments were carried out in triplicate.

The reaction mixtures, obtained after incubation of [14C]rofecoxib or 5-hydroxyrofecoxib with benzaldehyde, were analyzed by a Waters Q-ToF Ultima mass spectrometer operated in the positive and negative ion electrospray ionization mode for structural analysis. For obtaining the product ion spectra, argon gas was used as the collision gas, and the collision energy was set at 25 eV. The LC system used was a Waters Alliance 2695 separation module coupled with a 2996 photodiode array detector, scanned from 200 to 350 nm for 1 s. Chromatographic separations were carried out on an X Terra MS C18 column (2.1 × 150 mm, 5 μm) maintained at 30°C in a column oven. Solvent A and solvent B were at a flow rate of 0.2 ml/min. The mobile phase contained 10% solvent B in solvent A initially, and the proportion of solvent B was increased linearly to 70% in 30 min.

LC-NMR Analysis. A 200-μl aliquot of the reaction mixture, obtained after incubation of nonradiolabeled rofecoxib with benzaldehyde, was evaporated and reconstituted in 40 μl of 35% acetonitrile, and 20 μl of the reconstituted sample was subjected to LC-NMR analysis. On-flow LC-NMR analysis was performed using a Varian Inova 500 MHz NMR spectrometer equipped with a flow probe having an active volume of 60 μl. The LC system used was a Varian ProStar model 230 solvent delivery module and a model 310 UV-visible detector. An X Terra MS C18 column (2.1 × 150 mm, 5 μm) was isocratically eluted at an ambient temperature with CD3CN-D2O (35:65, v/v) at a flow rate of 0.2 ml/min. The NMR spectra were referenced to the signal of deuterated acetonitrile at 2.0 ppm. The residual solvent signals of deuterium oxide and deuterated acetonitrile were suppressed by water elimination through transverse gradients solvent suppression.

Radioactivity Measurement

Isolated aorta samples were measured for their wet weight, and each sample was solubilized with tissue solubilizer NCS-II (2 ml; GE Healthcare Bio-Sciences) under constant shaking at −55°C. Precipitated aortic homogenate samples were solubilized with 1 ml of NCS-II. After solubilization, these samples were mixed with 10 ml of liquid scintillator Hionic-Fluo (PerkinElmer Life and Analytical Sciences) and were subjected to radioactivity measurement by a liquid scintillation counter (model 2300TR; PerkinElmer Life and Analytical Sciences). The radioactivity in the aorta was calculated as an equivalent value of [14C]rofecoxib and expressed as a concentration per gram of aorta.

Histopathological Analysis

Aorta samples from the rats treated with rofecoxib (n = 6) or the vehicle (n = 6) were fixed using ½ Karnovsky’s solution in 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature. After the fixation, samples were processed and embedded in epoxy resin (TAAB Laboratories Equipment Ltd., Berkshire, UK). Sections were stained with tannic acid to demonstrate the elastic fibers (Cotta-Pereira et al., 1976), in addition to uranyl acetate and lead citrate staining, and were observed with a transmission electron microscope (type H7500; Hitachi Science System, Ibaraki, Japan).

Data Analyses

The area under the plasma concentration-time curve (AUC) to the last quantifiable time point (AUC_{<t}) was calculated using the computer program WinNonlin Professional (version 4.0.1; Pharsight Corporation, Mountain View, CA) with a noncompartment model. Statistical analysis of the experimental data were performed using an unpaired t test. Differences were considered to be significant when p < 0.05.
Results

Pharmacokinetics in Rats. After a single oral administration of rofecoxib (2 mg/kg) to the rats with or without treatment of SKF-525A (50 mg/kg), plasma concentrations of rofecoxib and 5-hydroxyrofecoxib were measured, and AUC\(_{0-t}\) values were calculated (Table 1). In a separate experiment, the rats with or without treatment of SKF-525A were dosed with [\(^{14}\)C]rofecoxib (2 mg/kg), and the radioactive concentrations in the aorta were measured (Table 1). Treatment with SKF-525A significantly decreased the AUC\(_{0-t}\) of only 5-hydroxyrofecoxib to one third of that without the treatment but did not decrease the AUC\(_{0-t}\) of rofecoxib. However, the treatment with SKF-525A caused no significant change in the concentration of radioactivity in the aorta.

In the experiment to examine the dose-response of pharmacokinetics, the rats were dosed orally with rofecoxib at a dose of 2, 5, or 10 mg/kg, and the AUC\(_{0-t}\) values of unchanged rofecoxib were determined. Other rats were dosed orally with [\(^{14}\)C]rofecoxib at a dose of 2, 5, or 10 mg/kg, and the concentrations of radioactivity in the aorta were determined. As shown in Fig. 1, there was a very good correlation (\(r^2 = 0.959\)) between the AUC\(_{0-t}\) of unchanged rofecoxib and the radioactive concentration in the aorta.

Covalent Binding of [\(^{14}\)C]Rofecoxib in Vitro to Rat Aorta Homogenate and Effect of Protein Modifiers. Homogenate of rat aorta, which had been pretreated with protein modifiers, D-penicillamine (10 mM), hydralazine (10 mM), BAPN (10 mM), or NaBH\(_4\) (20 mM), was incubated with [\(^{14}\)C]rofecoxib (100 \(\mu\)M) under a physiological pH condition (pH 7.4), and the radioactivity bound covalently to the proteins was measured (Fig. 2).

The radioactivity bound covalently to the aorta after incubation with the aortic homogenate without pretreatment with the protein modifiers was 12.9 ± 1.3 \(\mu\)g Eq/g (control). On the other hand, pretreatment of the aortic homogenate with the protein modifiers decreased the covalently bound aortic radioactivity to 15 to 40\% of the control. Radiodetection-HPLC analysis of the deproteinated supernatant fraction demonstrated that the major component is the unchanged rofecoxib (>90\%, data not shown). In a separate experiment, hydralazine, BAPN, and NaBH\(_4\) all significantly decreased the LOX activity to 0, 19, and 63\% of the control, respectively, whereas rofecoxib did not decrease it at all. The LOX activity in the presence of D-penicillamine could not be measured because the fluorescence in the assay mixture was too strong, perhaps due to its own fluorescence and/or the production of some unknown compound(s) with fluorescence.

Condensation Reaction of Rofecoxib with Benzaldehyde as a Model Reaction. [\(^{14}\)C]Rofecoxib (100 \(\mu\)M) was incubated with benzaldehyde (1 mM) in the phosphate buffer under a physiological pH condition (pH 7.4), and the reaction mixture was analyzed by radiodetection-HPLC. As shown in Fig. 3, in addition to the unchanged rofecoxib (eluted at 13.4 min), peaks of two radioactive components designated as PA and PB were observed at 15.6 and 16.4 min, respectively. The peak areas of PA and PB were increased with time during the incubation. At 24 h, the sum of the peak areas of PA and PB accounted for ~30\% of the total radioactivity chromatographed. No PA or PB was observed in the mixture in the absence of benzaldehyde.

The chemical structures of PA and PB were analyzed by LC/MS (Fig. 4). Both PA and PB exhibited the protonated molecule ion [M + H]\(^+\) at \(m/z\) 421, demonstrating that the molecular weight is greater than that of rofecoxib (314) by a mass of benzaldehyde (106). As shown in Fig. 4, PA and PB showed the same mass spectra, indicating that PA and PB are diastereomers. By analyzing the product ion spectra and fragmentation schemes for PA and PB, both PA and PB were proposed to be covalent adducts of rofecoxib with benzaldehyde formed by a condensation reaction. On the other hand, no reaction product was detected by LC/MS after incubation of 5-hydroxyrofecoxib with benzaldehyde.

Because PA and PB interconverted each other after isola-
tion by HPLC (data not shown), we determined the NMR spectra of PA and PB using an on-flow LC-NMR technique. Integration of the 1H NMR resonances of both PA and PB (Fig. 5) established 14 aromatic protons (6.99–7.72 ppm for PA and 7.20–7.78 ppm for PB), a pair of weak doublets corresponding to H_a and H_b (6.09 and 5.12 ppm for PA and 5.88 and 4.86 ppm for PB), and a methyl proton (3.16 ppm for PA and PB), clearly demonstrating that PA and PB are covalent adducts of rofecoxib with benzaldehyde.

**Histopathological Analysis.** The histopathological changes of the elastic fiber system of thoracic aorta after a 4-week multiple oral administration of rofecoxib (10 mg/kg)
to rats were evaluated by transmission electron microscopy (representative electron micrographs in Fig. 6). In the control (Fig. 6A), the elastic lamellae of all samples (six of six) appeared as continuous thick bands and were regularly parallel-arranged, among which many smooth muscle cells were found easily. On the other hand, in the rofecoxib-treated aorta (Fig. 6B), disruption and swelling of the elastic lamellae were noticed (six of six). Between the irregularly branching elastic lamellae, the smooth muscle cells were decreased in number and focally replaced by an abnormally large amount of collagenous fibers.

Discussion

In our previous study (Oitate et al., 2006), we demonstrated that the radioactivity from [14C]rofecoxib was covalently bound to the arterial elastin after oral administration to rats, but we could not identify the reactive components, i.e., whether rofecoxib or its metabolite was involved in the binding. In rats after administration of rofecoxib, the main components in plasma were reported to be unchanged rofecoxib, 5-hydroxyrofecoxib that was partially produced from the parent by some isoforms of cytochrome P450, and its glucuronide (5-hydroxyrofecoxib-O-β-D-glucuronide) (Halpin et al., 2000, Slaughter et al., 2003). Among them, the glucuronide was thought to be chemically inactive, so we hypothesized that either rofecoxib or 5-hydroxyrofecoxib or both were bound to the elastin. As shown in Table 1, the treatment of rats with SKF-525A, a nonspecific inhibitor of cytochrome P450, significantly decreased the systemic exposure (AUC₀₋₄₅₀) of 5-hydroxyrofecoxib with no statistical change in that of rofecoxib, whereas no significant change in the concentration of radioactivity retained by the aorta was observed, indicating that rofecoxib itself is bound covalently to elastin. Supporting this idea, there was a very good correlation between the systemic exposure of unchanged rofecoxib and the aortic radioactive concentration (Fig. 1).

The elastic property of elastin is due to the existence of covalent cross-linkages (Vrhovski and Weiss, 1998). The first step in this cross-linking is the formation of a highly reactive aldehyde, allysine, through oxidation of lysyl e-amino groups in elastin by LOX. Once formed, the aldehyde side chain of allysine is thought to react chemically with the aldehyde groups of other allysine molecules via an aldol condensation reaction or with e-amino groups of an unoxidized lysine residues via a Schiff’s base formation producing the tetrafunctional cross-linkages demosine and isodemosine (Fig. 7A). It has been demonstrated that the prevention of this cross-linking in elastin causes serious
lesions of connective tissues in animals and humans (Herd and Orbison, 1966; Andrews et al., 1975; Hashimoto et al., 1981; Junker et al., 1982; Light et al., 1986; Yoshikawa et al., 2001).

As shown in Fig. 2, the covalent binding of [14C]rofecoxib to aortic homogenate in vitro was significantly decreased by pretreatment of the homogenate with D-penicillamine or hydralazine, which has been reported to react with protein aldehydes in connective tissues and to form a thiazolidine or hydrazone analog (hydralazine is also able to inhibit LOX activity) (Pinnell et al., 1968; Deshmukh and Nimni, 1969; Gallop and Paz, 1975; Numata et al., 1981; Howard-Lock et al., 1986). BAPN, a specific inhibitor of LOX (Tang et al., 1983), also significantly decreased the binding, probably via the prevention of allysine production from lysine or by the reduction of a number of aldehyde groups of allysine by reacting chemically with aldehyde groups through Schiff's base formation. In addition, reductive pretreatment of aortic homogenate with NaBH₄, significantly blocked the binding, quite conceivably due to all existing aldehyde groups of allysine being reduced to alcohol. Regarding hydralazine and NaBH₄ having a LOX inhibitory activity, they might decrease the binding by the prevention of allysine production, the same as BAPN, in addition to the above-mentioned mechanisms. From these results, it was strongly suggested that the aldehydic functional group in elastin, i.e., the aldehyde group of allysine, is relevant to the covalent binding with rofecoxib.

These findings were also strongly supported by the other in vitro experiment using benzaldehyde as an aldehyde model compound. In this experiment, rofecoxib, but not 5-hydroxyrofecoxib, formed covalent adducts with benzaldehyde under a physiological pH condition, presumably by a condensation reaction (Figs. 3, 4, and 5). The C5 position in rofecoxib was considered to be sufficiently nucleophilic to react with aldehyde under a physiological pH condition (Fig. 4). This is consistent with the fact that 5-hydroxyrofecoxib, in which the C5 position is not nucleophilic because of hydroxylation, did not react with benzaldehyde.

To make it clear whether the covalent binding of rofecoxib to the allysine aldehyde has any effects on the arterial function, histopathological changes in the aortic wall were evaluated ultrastructurally after a 4-week multiple oral administration of rofecoxib to rats (Fig. 6). In the rofecoxib-treated group, the elastic lamellae in the tunica media of the thoracic aorta were focally disrupted, resulting in a greater degree of branching than those in the control aorta. These observations are similar to the findings in animals and humans under conditions inhibiting cross-linking of elastin fibers (Herd and Orbison, 1966; Andrews et al., 1975; Hashimoto et al., 1981; Light et al., 1986; Yoshikawa et al., 2001). In our previous report (Oitate et al., 2006), we demonstrated that the radioactivity from [14C]rofecoxib was localized on the elastic fibers of rat aortas by microautoradiography and that the accumulation of radioactivity in the aortas increased in a dose-frequency-dependent manner. Histopathological changes in the rat aortas after a 2-week treatment of rofecoxib was investigated in addition to the 4-week treatment, and we found that the degree of elastin degradation was also dose-frequency-dependent (data not shown), suggesting that the degree of rofecoxib-binding correlated with the degree of elastin degradation. It is thought to be possible that a degradation of elastic fibers similar to that observed in the aortas also occurs in the coronary arteries and the cerebral arteries, in which elastin also plays an important role in maintaining elasticity. Heart attacks and strokes, which were observed in the clinical cases of rofecoxib administration, might be evoked as a consequence of dysfunction of these arteries.

In conclusion, we propose the mechanism for the covalent binding of rofecoxib with rat elastin as schematically shown in Fig. 7. Under normal conditions (Fig. 7A), lysine in elastin is converted to allysine by LOX, and the subsequent spontaneous condensation between allysine and lysine residues produces further cross-linkages, desmosine and isodesmosine. However, rofecoxib reacts with the aldehyde group of allysine to give a condensation covalent adduct, leading to the prevention of normal cross-linking (Fig. 7B), which was considered to cause the degradation of elastic fibers as a consequence (Fig. 6). Because allysine is

![Fig. 6. Representative elastic fibers of the aortic media after a 4-week multiple administration of rofecoxib (10 mg/kg) to rats. Transmission electron microscopy with tannic acid stain. Original magnification, 2300X. A, control (vehicle); B, rofecoxib treatment.](https://jpet.aspetjournals.org/content/1201/6/1201/F6.large.jpg)
commonly present in the arteries in experimental animals and humans, it is presumed that in clinical situations the rofecoxib-induced degradation of elastic fibers in the arteries would lead to a dysfunction of the arteries and to further increased risk of CV events.

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