Celecoxib Decreases Endothelial Tissue Factor Expression Through Inhibition of c-Jun Terminal NH₂ Kinase Phosphorylation

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Background—Despite potential antiinflammatory properties, the use of selective cyclooxygenase-2 inhibitors (coxibs) in patients with cardiovascular diseases has been questioned because of a possibly increased thrombotic risk. Tissue factor (TF), a key protein for initiation of coagulation, has been implicated in the pathogenesis of atherosclerosis and thrombosis. Hence, we examined the effect of different coxibs on TF expression.

Methods and Results—Celecoxib (10⁻⁵ mol/L), but not rofecoxib (10⁻⁷ to 10⁻⁵ mol/L) or the experimental coxib NS-398 (10⁻⁷ to 10⁻⁵ mol/L), decreased tumor necrosis factor-α–induced TF expression and activity in human aortic endothelial cells. Celecoxib (10⁻⁵ mol/L) reduced activation of c-jun terminal NH₂ kinase (JNK), whereas it did not affect p38 mitogen-activated protein (MAP) kinase or p44/42 MAP kinase; in contrast, JNK activation was not affected by rofecoxib (10⁻⁵ mol/L) or NS-398 (10⁻⁵ mol/L). TF expression was reduced in a concentration-dependent manner by pretreatment with SP600125 (10⁻⁷ to 10⁻⁶ mol/L), a specific inhibitor of JNK, which confirms that JNK regulates tumor necrosis factor-α–induced TF expression.

Conclusions—Celecoxib reduced TF expression and activity in human aortic endothelial cells. Because neither rofecoxib nor the experimental coxib NS-398 affected TF expression, this effect occurs independently of COX-2 inhibition; it is rather mediated through inhibition of JNK phosphorylation. These data indicate a distinct heterogeneity within this class of drugs, which may be clinically relevant, especially for patients with atherosclerotic vascular diseases. (Circulation. 2005;111:1685-1689.)

Key Words: atherosclerosis ■ cardiovascular diseases ■ coagulation ■ endothelium ■ signal transduction

Cyclooxygenase-2 (COX-2) is expressed and activated at sites of inflammation, such as atherosclerotic plaques.¹,² The widespread use of selective COX-2 inhibitors (coxibs), which do not inhibit the COX-1 isoenzyme at therapeutic concentrations in humans, has been questioned because of concerns that relatively unopposed COX-2 inhibition may lead to an increased thrombotic risk.³,⁴ This is of great clinical interest, particularly in view of the large number of patients with arthritis; indeed, most of these patients have a relatively high incidence of comorbidities, including hypertension, diabetes, and atherosclerosis, which places them at considerable cardiovascular risk. Nevertheless, uncertainty persists because interpretation of observational studies is limited because of their retrospective design, selection biases, and uncontrolled nonuse of aspirin in higher-risk subsets.

Tissue factor (TF), a 263-residue membrane-bound glycoprotein, is a key enzyme for initiation and propagation of thrombus formation. TF plays an important role in atherogenesis; its expression is upregulated by inflammatory mediators such as tumor necrosis factor-α (TNF-α) and can be detected in a variety of cell types in atheromatous plaques.³,⁴ Furthermore, elevated levels of TF antigen and activity have been detected in plasma and atherectomy specimens of patients with unstable angina.⁵,⁶ Thus, considerable evidence suggests that TF is involved in vascular inflammation, which promotes atherosclerotic vascular diseases and acute coronary syndromes in particular; however, the effect of coxibs on TF expression has not yet been investigated. Therefore, the objective of this study was to evaluate the impact of the 2 most widely prescribed coxibs, celecoxib and rofecoxib, and of an experimental COX-2 inhibitor, NS-398, on endothelial TF expression and activity.
Methods

Cell Culture
Human aortic endothelial cells (Clonetics, Allschwil, Switzerland) were cultured as described previously. Cells were grown to confluence in 6-cm culture dishes and rendered quiescent for 24 hours before stimulation with 10 ng/mL TNF-α (Sigma) for 5 hours. Celecoxib (a gift from Pfizer), rofecoxib (a gift from Merck), or the experimental coxib NS-398 (Cayman Chemicals) was added 30 minutes before stimulation. SP600125, a specific inhibitor of c-jun N-terminal NH2 kinase (JNK; Calbiochem), was added 60 minutes before stimulation. To assess cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase was used according to the manufacturer’s recommendations (Roche).

Western Blot Analysis
Protein expression was determined by Western blot analysis. Cells were lysed in 50 mM Tris buffer as described previously. Forty-microgram samples were loaded and separated by 10% SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore) by semidry transfer. Equal loading was confirmed by Ponceau S staining. Antibody to human TF (American Diagnostica) was used at 1:2000 dilution; antibodies against phosphorylated p38 mitogen-activated protein (MAP) kinase (p38), phosphorylated p44/42 MAP kinase (ERK), and phosphorylated JNK (all from Cell Signaling) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, total ERK, and total JNK (all from Cell Signaling) were used at 1:2000, 1:10 000, and 1:1000 dilution, respectively. Blots were normalized to α-tubulin expression (1:10 000 dilution, Sigma). Representative blots are shown; bars represent at least 3 different experiments.

TF Activity
TF activity was analyzed with a colorimetric assay (American Diagnostica) according to the manufacturer’s recommendations. TF/factor VIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. Lipidated human TF was used as a positive control to confirm that the results obtained were in the linear range of detection (data not shown).

Statistical Analysis
Data are presented as mean±SEM. Statistical analysis was performed by 2-tailed unpaired Student t test. A probability value <0.05 was considered significant.

Results
TNF-α (10 ng/mL) induced TF expression in human aortic endothelial cells 30-fold compared with basal level (Figure 1). Celecoxib (10⁻⁷ to 10⁻⁴ mol/L) reduced TNF-α–induced TF expression in a concentration-dependent manner (Figure 1A); inhibition at 10⁻⁵ mol/L accounted for a decrease by 50% (P<0.001). Similarly, celecoxib (10⁻⁴ mol/L) decreased TNF-α–induced TF activity by 32% compared with stimulation with TNF-α alone (P<0.0001; Figure 1B). When higher concentrations of celecoxib, such as 3×10⁻⁵, were examined, TF expression was reduced even further; however, at these concentrations, a significant increase in cytotoxicity was observed (>5%). To exclude a nonspecific effect due to cytotoxicity, we confined our study to 10⁻⁵ mol/L celecoxib.
as the highest concentration. Two other coxibs, rofecoxib (10^{-7} to 10^{-5} mol/L) and NS-398 (10^{-7} to 10^{-5} mol/L), had no inhibitory effect on TNF-α–induced TF expression (P=NS; Figures 1C and 1D).

The MAP kinases p38, ERK, and JNK were transiently activated by TNF-α (10 ng/mL; Figure 2A). Celecoxib (10^{-5} mol/L) significantly decreased maximal phosphorylation of JNK compared with TNF-α alone (P<0.01; Figures 2A and 2B). A, TNF-α leads to transient, time-dependent activation of JNK (upper panel). Pretreatment with celecoxib (10^{-5} mol/L) reduces JNK phosphorylation, whereas phosphorylation of p38 (middle panel) and ERK (lower panel) is not altered. Expression of total JNK, p38, and ERKs remain unchanged. Pho indicates phosphorylated. B, After 15 minutes of stimulation with TNF-α, celecoxib reduces maximal activation of JNK but not p38 or ERK. *P<0.01 vs stimulation with TNF-α alone. Data are presented as percent MAP kinase phosphorylation compared with stimulation with TNF-α alone. C, SP600125, a specific inhibitor of JNK, impairs TNF-α–induced TF expression. *P<0.0001 vs stimulation with TNF-α alone. Blots are normalized to α-tubulin (αT) expression. Representative blots are shown; bars represent at least 3 different experiments.
NS-398 did not show any effect on TF expression or JNK phosphorylation. Because rofecoxib and the experimental coxib inhibitor SP600125 impaired TF expression in response to TNF-α alone (P<0.0001; Figure 2C). Unlike celecoxib, neither rofecoxib (10⁻⁵ mol/L) nor NS-398 (10⁻³ mol/L) significantly affected JNK phosphorylation (P=NS; Figures 3A and 3B). No significant increase in lactate dehydrogenase release was observed for any concentration of the drugs used (P=NS; data not shown) except for celecoxib concentrations above 10⁻⁵ mol/L.

Figure 3. Rofecoxib and NS-398 do not alter TNF-α-induced JNK activation. A and B, TNF-α leads to transient, time-dependent activation of JNK. After 15 minutes of stimulation, preincubation with rofecoxib or NS-398 does not alter TNF-α-induced JNK phosphorylation. P=NS vs stimulation with TNF-α alone. Rofe indicates rofecoxib; NS, NS-398. Blots are normalized to α-tubulin (αT) expression. Representative blots are shown; bars represent at least 3 different experiments.

2B). In contrast, activation of p38 or ERK was not affected by celecoxib (Figures 2A and 2B). Total expression of p38, ERK, or JNK also remained unaltered (Figure 2A). Pretreatment with SP600125 (10⁻⁷ to 10⁻⁴ mol/L), a specific inhibitor of JNK activation, decreased TF expression in a concentration-dependent manner to 42% compared with stimulation with TNF-α alone (P<0.0001; Figure 2C). Unlike celecoxib, neither rofecoxib (10⁻⁵ mol/L) nor NS-398 (10⁻³ mol/L) significantly affected JNK phosphorylation (P=NS; Figures 3A and 3B). No significant increase in lactate dehydrogenase release was observed for any concentration of the drugs used (P=NS; data not shown) except for celecoxib concentrations above 10⁻⁵ mol/L.

Discussion

The present study shows that celecoxib, but not rofecoxib or NS-398, decreases endothelial TF expression and activity. Expression of encrypted TF may account for the observation that inhibition of TF activity is less pronounced than the decrease in protein expression.¹³

TNF-α is a potent inducer of TF expression⁶,¹⁴; indeed, both TNF-α and TF are expressed in atherosclerotic plaques.¹,⁷,¹⁵ Therefore, we used this cytokine to mimic the inflammatory environment of the vessel wall for assessment of the effect of coxibs on TF expression. The MAP kinases JNK, p38, and ERK are involved in regulating TF expression in vascular cells in response to several stimuli.⁵,⁶,¹⁴ Consistent with this observation, all 3 MAP kinases were transiently activated in human aortic endothelial cells after stimulation with TNF-α. TNF-α-induced JNK activation was reduced by celecoxib but not by rofecoxib or NS-398. This observation is consistent with recent findings that celecoxib inhibits JNK activation in cancer cells.¹⁶ Inhibition of JNK by its specific inhibitor SP600125 impaired TF expression in response to TNF-α, which confirms the regulatory role of this MAP kinase in TNF-α-induced TF expression. We therefore conclude that the inhibitory effect of celecoxib on JNK phosphorylation mediates the reduction in TF expression. Whether JNK represents the direct intracellular target of celecoxib or whether it reduces TF expression through another mediator is unknown. Because rofecoxib and the experimental coxib inhibitor NS-398 did not show any effect on TF expression or JNK activation, the effect of celecoxib on TF expression and activity appears to occur independently of COX-2 inhibition.

Indeed, the concentrations used in the present study extend well above and below the IC₅₀ for inhibition of recombinant human COX-2, which are 4.0×10⁻⁸ mol/L, 3.4×10⁻⁷ mol/L, and 1.77×10⁻⁶ mol/L for celecoxib, rofecoxib, and NS-398, respectively.¹⁷,¹⁹

There is growing uncertainty about potentially harmful effects of coxibs in patients with cardiovascular diseases.²⁰,²⁰a The reduction in TF expression by celecoxib, but not rofecoxib or NS-398, adds to the evidence of an important heterogeneity within this class of drugs. Indeed, several preclinical studies demonstrated antiproliferative effects and cell cycle regulating functions of celecoxib in tumor cells and in endothelial cells that occurred independently of COX-2 inhibition.²¹–²³ Moreover, celecoxib but not rofecoxib significantly improved endothelial dysfunction.²⁴,²⁴a A consistent class effect of coxibs has been questioned further by recently published large-scale population-based cohort and case-control studies, which demonstrated that the odds of developing hypertension,²⁵ congestive heart failure,²⁶ and acute myocardial infarction²⁷ were not affected by celecoxib but increased in patients taking rofecoxib. Importantly, this issue was further highlighted by a recently published Food and Drug Administration–sponsored study in 1.3 million patients taking coxibs that showed striking differences within this widely prescribed class of drugs, with an increased risk of myocardial infarction in rofecoxib users in particular.²⁸

The role of TF in the development of cardiovascular diseases is increasingly recognized.⁷,²⁹ TF levels are elevated in patients with hypertension, dyslipidemia, diabetic vasculopathy, peripheral artery disease, and acute coronary syndromes.⁷,²⁹–³³ Impaired TF expression by celecoxib might represent a mechanism for the observed differences between celecoxib and other coxibs in patients with cardiovascular diseases. Hence, the present study adds to the evidence of a distinct heterogeneity within this widely prescribed class of drugs, which might have therapeutic implications for the treatment of patients with cardiovascular diseases.

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