Oxidant Stress and Aspirin-Insensitive Thromboxane Biosynthesis in Severe Unstable Angina

Francesco Cipollone, MD; Giovanni Ciabattoni, MD; Paola Patrignani, PhD; Massimo Pasquale, MD; Domenico Di Gregorio, MD; Tonino Bucciarelli, MD; Giovanni Davì, MD; Franco Cucurullo, MD; Carlo Patrono, MD

Background—Unstable angina is associated with enhanced lipid peroxidation and reduced antioxidant defenses. We have previously reported aspirin failure in the suppression of enhanced thromboxane (TX) biosynthesis in a subset of episodes of platelet activation in this setting. We tested the hypothesis that the in vivo formation of the F₂-isoprostane 8-iso-prostaglandin (PG)F₂α, a bioactive product of arachidonic acid peroxidation, is enhanced in unstable angina and contributes to aspirin-insensitive TX biosynthesis.

Methods and Results—Urine samples were obtained from patients with unstable angina (n=32), stable angina (n=32), or variant angina (n=4) and from 40 healthy subjects for the measurement of immunoreactive 8-iso-PGF₂α and 11-dehydro-TXB₂. 8-Iso-PGF₂α excretion was significantly higher in patients with unstable angina (339±122 pg/mg creatinine) than in matched patients with stable angina (236±83 pg/mg creatinine, P=0.001) and control subjects (192±71 pg/mg creatinine, P<0.0001). In patients with unstable angina, 8-iso-PGF₂α was linearly correlated with 11-dehydro-TXB₂ excretion (ρ=0.721, P<0.0001) and inversely correlated with plasma vitamin E (ρ=-0.710, P=0.004). Spontaneous myocardial ischemia in patients with variant angina or ischemia elicited by a stress test in patients with stable angina was not accompanied by any change in 8-iso-PGF₂α excretion, thus excluding a role of ischemia per se in the induction of increased F₂-isoprostane production.

Conclusions—These findings establish a putative biochemical link between increased oxidant stress and aspirin-insensitive TX biosynthesis in patients with unstable angina and provide a rationale for dose-finding studies of antioxidants in this setting. (Circulation. 2000;102:1007-1013.)

Key Words: angina • aspirin • isoprostanes • thromboxane • oxidant stress

The association among oxidative modifications of LDL,1,2 reduced antioxidant defenses,3 and unstable angina has been well documented. Moreover, numerous studies have shown that the dietary intake of vitamin E is inversely correlated with the risk of cardiovascular disease4 and that vitamin E supplementation reduced nonfatal coronary events in a randomized clinical trial in patients with coronary artery disease.5

Recently, a series of bioactive prostaglandin (PG)F₂-like compounds (isoprostanes) have been discovered6 that are produced from arachidonic acid through a nonenzymatic process of lipid peroxidation, catalyzed by oxygen free radicals on cell membranes and LDL particles. Among these products, of particular interest is 8-iso-PGF₂α (also known as iPF₂α-III), which induces vasoconstriction and amplifies the response of human platelets to other agonists (for a review, see Patrono and FitzGerald7). F₂-isoprostanes can be reliably measured in both plasma and urine8,9 and have been shown to be increased in association with cigarette smoking,10,11 diabetes mellitus,12 and hypercholesterolemia.13,14 Consistent with the hypothesis of a contribution of 8-iso-PGF₂α to persistent platelet activation in these settings, dose-dependent suppression of F₂-isoprostane formation by vitamin E supplementation was associated with significant reductions in 11-dehydro-thromboxane (TX)B₂ (TXM) excretion in diabetic12 and hypercholesterolemic13 patients.

Episodic increases in TXA₂ biosynthesis have been reported in patients with unstable angina.15,16 Enhanced TX biosynthesis in this setting is likely to reflect episodes of platelet activation, because it was largely suppressed with low-dose aspirin.16 However, despite >95% suppression of the cyclooxygenase (COX) activity of platelet prostaglandin H synthase (PGHS-1) by aspirin, incomplete suppression of TXM excretion has been detected in some patients with unstable angina.16

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From the Departments of Medicine and Aging (F. Cipollone, P.P., M.P., G.D., F. Cucurullo, C.P.) and Biomedical Sciences (T.B.), University of Chieti “G. D’Annunzio” School of Medicine, Chieti, Italy; Department of Pharmacology (G.C.), Catholic University School of Medicine, Rome, Italy; and the Division of Cardiology (D. Di G.), “F. Renzetti” Hospital, Lanciano, Italy.

Correspondence to Carlo Patrono, MD, Dipartimento di Medicina e Scienze dell’Invecchiamento, Università degli Studi “G. D’Annunzio,” Via dei Vestini 31, 66013 Chieti, Italy. E-mail cpatrono@unich.it

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Baseline Characteristics of Study Patients With Ischemic Heart Disease

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stable Angina</th>
<th>Unstable Angina</th>
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<tr>
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<td>Previous infarction</td>
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<td>Aspirin</td>
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We speculated that increased oxidant stress in unstable angina could induce the generation of 8-iso-PGF<sub>2α</sub> and other biologically active isoeicosanoids and that these compounds could in turn contribute to aspirin-insensitive TX biosynthesis in this setting. Therefore, in the present study, we investigated whether 8-iso-PGF<sub>2α</sub> formation is altered in patients with severe unstable angina compared with matched patients with stable angina and healthy subjects and whether it correlates with the rate of TXA<sub>2</sub> biosynthesis. Additional studies were performed to assess the influence of myocardial ischemia per se and COX-1 or -2 inhibition on 8-iso-PGF<sub>2α</sub> and TXA<sub>2</sub> biosynthesis.

Methods

Subjects
Between February 1993 and June 1998, we studied 32 patients with severe unstable angina (Braunwald class III<sup>B</sup>), 32 patients with chronic stable angina, 4 patients with active variant angina, and 40 healthy volunteers. None of the participating subjects were taking any antiplatelet drug at the time of study due to contraindications. The study protocol was approved by the institutional ethics committee. The baseline clinical characteristics of patients are given in the Table.

Study Design
We conducted a cross-sectional comparison of urinary 8-iso-PGF<sub>2α</sub> and TXM in patients with unstable angina (n=20) or stable angina (n=20) and 40 age- and gender-matched control subjects (22 men and 18 women, mean age 54±8 years). Moreover, patients with stable and unstable angina were also matched for hypercholesterolemia, diabetes, cigarette smoking, hypertension, and drug therapy to minimize potential confounders (Table). Each patient underwent an overnight urine collection. Urine samples were added with the antioxidant 4-hydroxy-tempo (1 mmol/L; Sigma Chemical Co) and stored at −20°C until extraction.

To assess the potential influence of myocardial ischemia on F<sub>2</sub>-isoprostane and TX biosynthesis, 2 additional studies were performed. In the first, 12 men with stable angina (mean age 62±7 years) were studied. On admission, these patients were monitored with Holter monitoring during the first day of hospitalization to exclude the presence of spontaneous myocardial ischemia. Patients were subjected to an exercise stress test with bicycle ergometry during continuous 12-lead ECG monitoring to assess the relation of 8-iso-PGF<sub>2α</sub> excretion to the occurrence of myocardial ischemia. Four consecutive 6-hour urine samples were collected during the first 24 hours of study, and 2 consecutive urine samples (at 30 minutes and 6 hours, respectively) were collected after exercise-induced myocardial ischemia. In the second study, we measured 8-iso-PGF<sub>2α</sub> in 46 urine samples collected from 4 patients with active variant angina during 96 hours of observation.

Because small amounts of 8-iso-PGF<sub>2α</sub> can be formed by human platelets and monocytes through a COX-1– and -2–dependent mechanism,7 respectively, we also evaluated whether the inhibition of COX had any influence on 8-iso-PGF<sub>2α</sub> excretion. Thus, to explore the potential contribution of platelet COX-1, we measured 8-iso-PGF<sub>2α</sub> and TXM in 47 urine samples collected from 8 patients with stable angina (mean age 62±7 years) taking low-dose aspirin (100 mg/d) and compared it with 17 samples obtained from 4 patients with stable angina (mean age 63±7 years) who were not taking any antiplatelet drug at the time of study due to contraindications. To evaluate the potential contribution of monocyte COX-2, 12 patients with unstable angina (6 men and 6 women, mean age 64±11 years) were randomly assigned to receive (1) conventional therapy plus 1 mg/kg 6-methyl-prednisolone (6-MP) BID for 2 days, administered in a 25-minute intravenous infusion (n=6) or (2) conventional therapy plus placebo (as 0.9% NaCl solution) BID for 2 days (n=6). All patients received 100 mg/d aspirin during the study. Twelve 6-hour urine samples were collected from each patient, and a total of 144 urine samples were analyzed for 8-iso-PGF<sub>2α</sub> and TXM.

To investigate the relationship between endogenous plasma antioxidants and F<sub>2</sub>-isoprostane biosynthesis, blood and urine samples were obtained from 14 patients with severe unstable angina (7 men and 7 women, mean age 54±9 years) after a 12-hour fast for the determination of plasma vitamin E and urinary 8-iso-PGF<sub>2α</sub>. Blood was drawn into test tubes that contained EDTA (2.7 mmol/L) and were separated within 1 hour after sampling. Vitamin E plasma content was determined with HPLC.<sup>18</sup>

Urinary Eicosanoid Assays
Urinary 8-iso-PGF<sub>2α</sub> and TXM levels were measured according to previously described and validated radioimmunoassay methods.<sup>9,19</sup>

Statistical Analysis
For the clinical data, variables were compared with the use of the χ<sup>2</sup> test. The biochemical data were analyzed according to nonparametric methods. An ANOVA was performed with the Kruskal-Wallis method. Subsequent pairwise comparisons were made with the Mann-Whitney U test with corrections for multiple comparisons. The differences between baseline and postprocedural values were analyzed with the Wilcoxon signed-rank test. Moreover, the association of eicosanoid measurements with other biochemical parameters was assessed with the Spearman rank correlation test. The number of ischemic episodes and the ischemic burden are expressed as median and range; the remaining variables are reported as mean±SD. Statistical significance was considered to be indicated by a P value of <0.05, except for multiple comparisons, where the threshold for statistical significance was defined on the basis of the number of comparisons.

Results
Urinary 8-iso-PGF<sub>2α</sub> excretion was significantly higher in patients with unstable angina (339±122 pg/mg creatinine, mean±SD, n=20) than in matched patients with stable angina (236±83 pg/mg creatinine, n=20, P=0.001) and in healthy subjects (192±71 pg/mg creatinine, n=40, P<0.0001) (Figure 1).
Patients with unstable angina taking low-dose aspirin had significantly higher TXM excretions than did patients with stable angina (532±227 versus 194±89 pg/mg creatinine, n=20, P<0.0001) (Figure 2). A statistically significant correlation was found between 8-iso-PGF$_{2\alpha}$ and TXM excretion in patients with unstable angina (ρ=0.721, P<0.0001) (Figure 3) but not in patients with stable angina or healthy subjects (not shown). Moreover, as shown in Figure 4, a statistically significant inverse correlation was found between plasma levels of vitamin E and urinary 8-iso-PGF$_{2\alpha}$ (ρ=−0.710, P=0.004) and TXM (ρ=−0.696, P=0.006) in patients with unstable angina.

**Effects of Myocardial Ischemia**

By investigating 8-iso-PGF$_{2\alpha}$ excretion before and after a positive exercise stress test in 12 patients with chronic stable angina, we sought to determine whether myocardial ischemia per se was responsible for triggering the enhanced biosynthesis of F$_2$ isoprostanes. Urinary 8-iso-PGF$_{2\alpha}$ did not increase as a result of myocardial ischemia, as reflected with values of 234±128 (n=48) before and 219±111 (n=24) pg/mg creatinine after effort-induced myocardial ischemia (P=NS) (Figure 5). Moreover, to exclude that the severity of ischemia caused by effort in stable patients could be insufficient to cause detectable lipid peroxidation, we also measured 8-iso-PGF$_{2\alpha}$ excretion in 4 patients with active variant angina. During the first 24 hours of Holter monitoring, all patients with variant angina and 23 of 32 patients with unstable angina had at least 1 ischemic episode. Both the number of ischemic episodes (median 8 and range 1 to 10 versus median 1 and range 0 to 3 per patient) and the total ischemic burden (median 39 and range 3 to 112 versus median 18 and range 0 to 63 minutes per patient) were greater in patients with variant...
angina than in patients with unstable angina. Despite this, the level of lipid peroxidation in patients with variant angina was comparable to that of patients with stable angina and lower than of patients with unstable angina. Moreover, in patients with variant angina, 8-iso-PGF₂α excretion did not differ between the samples collected during myocardial ischemia (249 ± 119 pg/mg creatinine, n = 19) and those collected during the ischemia-free periods (262 ± 129 pg/mg creatinine, n = 27).

Effects of COX Inhibition

Because relatively small amounts of 8-iso-PGF₂α can be formed enzymatically through the COX activity of platelet PGHS-1 and monocyte PGHS-2, we explored the effects of COX inhibition on the urinary excretion of 8-iso-PGF₂α. Thus, we used aspirin and 6-MP to investigate the mechanism or mechanisms of F₂-isoprostane formation in ischemic heart disease. Aspirin irreversibly acetylates the serine residue at position 529 (Ser529) in the polypeptide chain of PGHS-1.²⁰ By virtue of this unique mechanism of action, the daily intake of low doses of aspirin selectively suppresses platelet TXA₂ synthesis in a cumulative fashion.²¹ As shown in Figure 6, urinary 8-iso-PGF₂α excretion was not significantly different in patients with stable angina treated with low-dose aspirin versus those untreated with low-dose aspirin, despite a statistically significant difference in TXM excretion.

Previous in vitro evidence demonstrates that glucocorticoids can prevent inducible monocyte 8-iso-PGF₂α production in association with the suppression of PGHS-2 induction.²² The excretion rates of urinary 8-iso-PGF₂α measured before, during, and after 6-MP or placebo infusions are depicted in Figure 7. In patients randomized to receive 6-MP, 8-iso-PGF₂α formation did not show any statistically significant change between the first and the last day of study (252 ± 169 versus 215 ± 147 pg/mg creatinine). Moreover, no statistically significant differences were found between placebo and 6-MP treatment. These findings are consistent with a COX-independent mechanism of F₂-isoprostane formation in unstable angina.

Discussion

Previous studies have examined the role of lipid peroxidation in acute coronary syndromes through the measurements of malondialdehyde¹ or the susceptibility of the LDL of the patient to oxidation in vitro.² However, malondialdehyde is a byproduct of COX activity in platelets,³ thus resulting in lack of specificity in clinical syndromes characterized by platelet activation. Moreover, it is unclear whether the wide variations reported in the susceptibility of LDL from different individuals to oxidation ex vivo have any relevance to the actual extent of LDL oxidation in vivo.

In the present study, we used the urinary excretion of the F₂-isoprostane 8-iso-PGF₂α as a marker of in vivo lipid peroxidation.⁶,⁷ The measurement of this chemically stable compound has several distinct advantages over other markers of oxidant stress. (1) It reflects a nonenzymatic process of lipid peroxidation of an ubiquitous, endogenous substrate (ie, arachidonic acid) that is catalyzed by oxygen radicals.⁶ (2) Once released from cell membranes or LDL, 8-iso-PGF₂α circulates in peripheral venous blood.⁸ (3) Urinary excretion of this metabolite has been well characterized in humans.⁸,⁹ This analytical approach has been used previously to demonstrate enhanced lipid peroxidation in association with advanced age,⁹ cigarette smoking,¹⁰,¹¹ diabetes mellitus,¹² and hypercholesterolemia,¹³,¹⁴ as well as after coronary artery reperfusion.²⁴,²⁵

In the present study, we found that the formation and urinary excretion of 8-iso-PGF₂α are abnormally elevated
in the vast majority of patients with severe unstable angina who were carefully characterized for other variables that could influence in vivo lipid peroxidation (Table). This is the first report of enhanced in vivo lipid peroxidation in patients with unstable angina. Previously, Delanty et al reported elevated levels of 8-iso-prostaglandins in patients with acute myocardial infarction who were undergoing thrombolysis.

We further examined whether enhanced 8-iso-prostaglandin formation is a consequence of myocardial ischemia. The studies in patients with stable angina and active variant angina argue against this possibility by showing substantially unchanged rates of 8-iso-PGF$_{2\alpha}$ excretion up to 6 hours after exercise-induced myocardial ischemia (Figure 5) and during persistent transmural ischemia induced by coronary artery spasm. Similarly, Reilly et al reported that patients undergoing coronary angioplasty for ischemic symptoms did not have elevated preprocedural levels of 8-iso-PGF$_{2\alpha}$.

Because platelet COX activity can be a source of relatively small amounts of 8-iso-PGF$_{2\alpha}$ compared with TXB$_2$ we asked whether enhanced urinary excretion of the former and its correlation with TXM excretion in unstable angina might simply reflect an ongoing process of platelet activation in this setting. This seems unlikely because (1) platelet COX-1 activity was blocked by aspirin treatment in all patients and (2) aspirin-insensitive TXM excretion in unstable angina is likely to reflect extrapolatelet sources of TXA$_2$ biosynthesis possibly driven by COX-2 induction. Moreover, the study in patients with chronic stable angina that compared metabolite excretion in the presence and absence of platelet COX blockade by low-dose aspirin clearly shows that the suppression of TXA$_2$ biosynthesis is not associated with any detectable change in F$_2$-isoprostane formation (Figure 6). These results are consistent with a platelet COX-independent mechanism of formation of 8-iso-PGF$_{2\alpha}$ in ischemic heart disease, as demonstrated in other clinical settings.

The induction of PGHS-2 in monocytes/macrophages in response to a local inflammatory/mitogenic milieu can provide a source of aspirin-insensitive TXA$_2$ biosynthesis in unstable angina, as suggested previously and confirmed in the present study. Moreover, the COX activity of monocyte PGHS-2 can also generate small amounts of 8-iso-PGF$_{2\alpha}$ in vitro. Thus, to exclude the contribution of an acute inflammatory reaction to enhanced 8-iso-PGF$_{2\alpha}$ formation in unstable angina, we exploited the capacity of glucocorticoids to suppress monocyte PGHS-2 induction in response to lipopolysaccharide, thereby preventing COX-2–dependent 8-iso-PGF$_{2\alpha}$ formation. The failure of intravenous 6-MP to suppress 8-iso-PGF$_{2\alpha}$ excretion in patients with unstable angina (Figure 7) argues against a COX-2–dependent mechanism of F$_2$-isoprostane formation in this setting.

Having established that enhanced excretion of 8-iso-PGF$_{2\alpha}$ is likely to reflect a nonenzymatic process of lipid peroxidation in patients with unstable angina, we examined the correlation between its rate of formation and plasma vitamin E levels. Vitamin E is a potent, lipid soluble antioxidant that is present in plasma and LDL. Our previous studies performed in hypercholesterolemic and diabetic patients demonstrated dose-dependent reductions in F$_2$-isoprostane formation in response to short-term vitamin E supplementation. Consistent with these results, we found a statistically significant inverse correlation between plasma vitamin E levels and 8-iso-PGF$_{2\alpha}$ excretion rates in patients with unstable angina (Figure 4). Thus, most likely, enhanced lipid peroxidation in patients with severe unstable angina reflects an altered oxidant/antioxidant balance. The mechanism or mechanisms and location of the oxidant species responsible for increased formation of F$_2$-isoprostanes remain unanswered in the present study because of the obvious limitations inherent to urinary measurements. The localization of distinct isoprostanes and other products of lipid peroxidation in carotid atherosclerotic lesions and their relationship to plaque instability suggest that a similar scenario may occur in coronary plaques.

In patients with unstable angina, ~50% of the individual variation in aspirin-insensitive TXA$_2$ biosynthesis could be accounted for by the individual variation in 8-iso-PGF$_{2\alpha}$ formation (Figure 3). Although a common factor (eg, acute inflammation) might trigger both aspirin-insensitive TXA$_2$ biosynthesis and nonenzymatic formation of F$_2$-
isoprostanes and thus contribute to the correlation between the 2, this seems unlikely in view of the intervention studies discussed earlier and of the correlation with endogenous vitamin E levels. A more likely explanation for this correlation between 8-iso-PGF$_{2\alpha}$a and TXM excretion, detectable in unstable angina but not in chronic stable angina, is that enhanced lipid peroxidation and formation of bioactive isoeicosanoids represent an important determinant of aspirin-insensitive TX biosynthesis. COX-2 induction in response to a variety of lipid mediators, including substrates, products, and inhibitors of PGH-synthase, has recently been described in mammary epithelial cells in vitro.32

Concentrations of 8-iso-PGF$_{2\alpha}$a in the range of 1 nmol/L to 1 µmol/L induce a dose-dependent increase in platelet shape change, calcium release from intracellular stores, and inositol phosphates.7 Moreover, 8-iso-PGF$_{2\alpha}$a increases platelet adhesion and reduces the antiadhesive and antiaggregatory effects of nitric oxide.33 Furthermore, 8-iso-PGF$_{2\alpha}$a causes dose-dependent, irreversible platelet aggregation in the presence of concentrations of collagen, ADP, arachidonic acid, and PGH$_2$/TXA$_2$; analogs that, when acting alone, fail to aggregate platelets.34 Although platelet-active concentrations of 8-iso-PGF$_{2\alpha}$a may not be achieved in circulating blood, it should be pointed out that this compound is only 1 of a series of biologically active isoeicosanoids formed through a similar non-COX mechanism of lipid peroxidation.35 In fact, up to 64 F$_2$-isoprostanes may be formed, and similar families of isomers of other prostaglandins and lipoxygenase and epoxyxygenase products are likely to be generated.35 This level of complexity constrains the interpretation of studies that focus on a single isomer, particularly when attempting to define their likely role as bioactive autacoids in vivo. Thus, both PGHS-2–derived TXA$_2$ and nonenzymatic F$_2$-isoprostane formation might represent 2 important ei-

cosanoid mechanisms that contribute to aspirin-insensitive platelet activation in unstable angina. The 2 mechanisms might play a variable role in different patients with unstable angina, depending on the prevalence of an acute inflammatory reaction and its endogenous modulation versus enhanced oxidant stress and its endogenous regulation.

We conclude that increased nonenzymatic formation of F$_2$-isoprostanes may provide an important biochemical link between an altered oxidant/antioxidant balance and aspirin-insensitive TX biosynthesis in patients with unstable angina. These results provide a rationale for dose-

founding studies of antioxidants in acute coronary syndromes, with F$_2$-isoprostane formation used as the primary biochemical end point.

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