Modulation of Aspirin-Insensitive Eicosanoid Biosynthesis by 6-Methylprednisolone in Unstable Angina

Francesco Cipollone, MD; Antonina Ganci, MD; Anita Greco, PhD; Maria Rosaria Panara, PhD; Massimo Pasquale, MD; Domenico Di Gregorio, MD; Ettore Porreca, MD; Andrea Mezzetti, MD; Franco Cuccurullo, MD; Paola Patrignani, PhD

Background—The evidence that inflammation plays a pivotal role in the pathophysiology of acute coronary syndromes prompted us to investigate the effects of glucocorticoid treatment on leukotriene (LT) C₄ and thromboxane (TX) A₂ biosynthesis in unstable angina.

Methods and Results—Urinary LTE₄ and 11-dehydro-TXB₂ were significantly higher in 12 patients with unstable angina than in 12 patients with stable angina and 12 patients with nonischemic chest pain. Furthermore, we randomized the unstable angina patients to receive intravenous 6-methylprednisolone (6-MP; 1 mg/kg BID for 2 days) or matching placebo and collected 12 consecutive 6-hour urine samples before and during the infusions. LTE₄ excretion showed a time-dependent decrease in the 6-MP group but did not decrease during placebo. Furthermore, during myocardial ischemia, LTE₄ was significantly higher before 6-MP infusion than during steroid therapy. In contrast, 11-dehydro-TXB₂ did not differ significantly during 6-MP versus placebo. Myocardial ischemia elicited by stress test in the stable angina patients was not accompanied by any change in LTE₄ and 11-dehydro-TXB₂, thus ruling out a role of ischemia per se in the induction of increased eicosanoid production.

Conclusions—Increased production of vasoactive LT and TX may occur in unstable angina despite conventional antithrombotic and antianginal treatment. Glucocorticoids can suppress LTC₄ biosynthesis in the short term and may provide an interesting tool to explore the pathophysiological significance of inflammatory cell activation in this setting. (Circulation. 2003;107:55-61.)

Key Words: angina ● inflammation ● prostaglandins ● leukocytes ● platelets

Although platelet activation plays a major role in the pathogenesis of acute coronary syndromes,¹ more recent demonstrations suggest that an acute inflammatory reaction may also contribute to these conditions.² Important eicosanoid mechanisms operate in unstable angina, as reflected by measurements of thromboxane (TX) A₂ biosynthesis and aspirin trials in this setting.³ Two important mechanisms of aspirin-insensitive formation of vasoactive eicosanoids have been characterized (ie, the formation of leukotrienes [LTs] by 5-lipoxygenase⁴ and TXA₂ production by cells expressing an inducible form of cyclooxygenase [COX]).⁵ LTC₄ and LTD₄ are potent arterial vasoconstrictors.⁶ Moreover, enhanced LT biosynthesis has been detected during the acute phase of unstable angina⁷ and during PTCA.⁸ COX-derived TXA₂ is a potent agonist of platelet aggregation and has vasoconstrictive properties.⁹ However, despite >95% suppression of platelet COX-1 by aspirin, incomplete suppression of TX metabolite excretion has been detected in unstable angina.¹⁰¹¹ Whereas mature platelets contain only constitutively expressed COX-1,¹² in other cells (such as endothelial and smooth muscle cells, monocytes, and newly formed platelets), a highly inducible isofom called COX-2 has been identified.¹³¹⁴¹⁵ Glucocorticoids are potent anti-inflammatory drugs¹⁶ and provide an important therapeutic approach to several diseases associated with chronic inflammation. Their mechanism of action, however, is not yet totally clear. Sebaldt et al¹⁷ demonstrated that steroids can suppress the release of arachidonic acid and inhibit eicosanoid biosynthesis in human macrophages ex vivo. Recently, Santini et al¹⁸ reported that the administration of 6-methylprednisolone (6-MP) to healthy subjects causes a dose-dependent inhibition of inducible prostanoid biosynthesis, presumably through down-regulation of monocyte COX-2 expression.

In the present study, we tested the hypothesis that neutrophil and monocyte activation may be responsible for the increase in LTC₄ and TXA₂ biosynthesis in aspirin-treated patients with unstable angina. Thus, we contrasted the effects...
of 6-MP on eicosanoid metabolite excretion with those of placebo in a randomized, double-blind study. Additional studies were performed to assess the influence of myocardial ischemia per se and platelet COX inhibition by low-dose aspirin on LTC4 and TXA2 biosynthesis.

**Methods**

**Subjects**

Twelve patients with unstable angina (class IIIB; 6 men and 6 women aged 64±11 years), 12 patients with stable angina (7 men and 5 women aged 64±9 years), and 12 with nonischemic chest pain patients (6 men and 6 women aged 54±16 years) were consecutively entered into the study. In addition, we studied 4 healthy staff members (1 man and 3 women aged 35±9 years). Informed consent was obtained from each subject. The Institutional Ethics Committee approved the study protocol. The baseline clinical characteristics of patients with angina are detailed in the Table.

**Unstable Angina Study Design**

Patients were randomly assigned to receive either IV 6-MP (1 mg/kg BID) for 2 days or a placebo (0.9% NaCl solution) twice a day for 2 days. Conventional therapy was allowed as required by clinical judgment. All patients were under antplatelet treatment at enrollment and received 100 mg/d aspirin during the study.

**Stable Angina Study Design**

Holter monitoring during the first day excluded the presence of spontaneous myocardial ischemia; after that, patients were subjected to a positive exercise stress test. Before the stress test, patients were randomly assigned to receive either a single dose of IV 6-MP (1 mg/kg) or a single dose of placebo (0.9% NaCl solution). Conventional therapy was allowed as required by clinical judgment.

**Control Groups**

Twelve hospitalized subjects who presented with nonischemic chest pain formed a first control group. Medical therapy was allowed as required by clinical judgment, but no patients received glucocorticoids or nonsteroidal anti-inflammatory agents. In addition, these patients received aspirin (100 mg/d) during the study. For a second control group, 4 healthy subjects volunteered to receive aspirin (100 mg/d) for 3 days.

**Blood Collection**

We collected 4 blood samples from each unstable angina patient at baseline and at 24, 48, and 72 hours after randomization.

**Urine Collection**

Twelve consecutive 6-hour samples were obtained from each unstable angina patient. In the stable angina group, we collected 4 consecutive 6-hour samples during the first 24 hours of study and 2 consecutive samples (at 30 minutes and 6 hours, respectively) after the exercise-induced myocardial ischemia. Single samples (5.8±1 hours) were obtained from each nonischemic patient. Finally, we collected 3 overnight samples (7.7±1 hours) from the healthy volunteers before and after aspirin administration. A total of 1 mmol/L 4-OH TEMPO (Sigma) was added to each urine sample, and samples were stored at −80°C until testing.

**Assays of Urinary LTE4 and 11-Dehydro-TXB2**

Urinary LTE4 was measured by high-performance liquid chromatography followed by radioimmunoassay as previously described. Urinary 11-dehydro-TX2 was measured by previously described and validated radioimmunoassay methods. All results were expressed as pg/mg creatinine. We tested 6-MP for cross-reactivity with the anti-LTE4 serum and with the anti-11-dehydro-TX2 serum and found no displacement of the tracers at 6-MP concentrations up to 1 μg/mL.

**Figure 1.** LTE4 (a) and 11-dehydro-TXB2 (b) measured before the treatment in patients with unstable angina (UA; n=12), stable angina (SA; n=12), and nonischemic chest pain (n=12) and in healthy subjects (n=4). Numbers in parentheses reflect the number of analyzed samples. The bars and vertical lines represent mean±SD values.
Statistical Analysis
For the clinical data, variables were compared with the χ² test. ANOVA was performed with the Kruskal-Wallis method. Subsequent pairwise comparisons were made with the Mann-Whitney U test. The differences between baseline and post-treatment values were analyzed with the Wilcoxon test. All values are reported as mean±SD. Statistical significance was considered to be indicated by P<0.05.

Results
Clinical Data
Ischemic events at rest were detected during 31 (22%) of 144 urine collection periods in the unstable angina patients. In the unstable angina patients randomized to 6-MP or placebo, no significant differences in blood cell counts were observed during the study.

Eicosanoid Biosynthesis
Urinary LTE₄, averaged 57±47 pg/mg creatinine (n=48) in the unstable angina patients. LTE₄ was higher compared with stable angina patients (32±12 pg/mg creatinine, n=48; P<0.02), patients with nonischemic chest pain (21±10 pg/mg creatinine, n=12; P=0.002), and healthy subjects (28±19 pg/mg creatinine, n=12; P<0.02; Figure 1a). The difference in LTE₄ between stable angina and nonischemic chest pain patients was also statistically significant (P=0.003; Figure 1a). Urinary 11-dehydro-TXB₂ averaged 365±475 pg/mg creatinine (n=48) in the unstable angina patients. It was higher compared with stable angina patients on aspirin therapy (153±103 pg/mg creatinine, n=32; P<0.05), aspirin-treated patients with nonischemic chest pain (78±41 pg/mg creatinine, n=12; P<0.0001), and healthy subjects after aspirin administration (26±19 pg/mg creatinine, n=12; P<0.0001; Figure 1b). Urinary 11-dehydro-TXB₂ was also higher in patients with stable angina compared with both those with nonischemic chest pain (P<0.02) and healthy volunteers (P<0.0001; Figure 1b).

Effects of Platelet COX Inhibition
In the healthy volunteers treated with aspirin, LTE₄ averaged 28±19 pg/mg creatinine before and 38±24 pg/mg creatinine after aspirin treatment (n=12, P=NS; Figure 2a). In contrast, 11-dehydro-TXB₂ decreased from 124±72 pg/mg creatinine to 26±19 pg/mg creatinine during aspirin treatment (n=12, P<0.0001; Figure 2a). Levels of 11-dehydro-TXB₂ were reduced by aspirin in a cumulative fashion by 59%, 72%, and 80% on the first, second, and third day of treatment, respectively. Similarly, in the patients with stable angina, there was no difference in LTE₄ between the 8 patients on aspirin therapy (33±13 pg/mg creatinine, n=32) and the 4 patients not taking any antiplatelet treatment (34±11 pg/mg creatinine, n=16), despite significantly lower 11-dehydro-TXB₂ concentrations (153±103 versus 441±299 pg/mg creatinine, P<0.0001; Figure 2b).

Eicosanoid Biosynthesis in Unstable Angina Patients Randomized to 6-MP or Placebo
LTE₄ excretion during the study is depicted in Figure 3. In patients treated with 6-MP (n=6), LTE₄ showed a time-dependent decrease from 90±55 pg/mg creatinine before 6-MP infusion to 15±9 pg/mg creatinine in the last urine collection. Thus, 6-MP was associated with a 76% suppression in LTE₄, from the first through the last day of the study (Figure 4). In patients randomized to placebo, LTE₄ did not spontaneously decrease as a function of time (Figure 3), and no changes were observed from the first through the last day of the study (53±43 versus 49±38 pg/mg creatinine; Figure 4).

The time course of 11-dehydro-TXB₂ is depicted in Figure 5. 11-dehydro-TXB₂ decreased in the 6-MP group from 363±99 pg/mg creatinine before 6-MP to 212±156 pg/mg creatinine during the last day of study. However, 11-dehydro-TXB₂ excretion spontaneously decreased as a function of time in the placebo group as well (367±312 versus 96±11 pg/mg creatinine). Thus, 11-dehydro-TXB₂ did not differ...
during 6-MP versus placebo to any statistically significant extent, although the large interindividual variability in metabolite excretion rates would preclude detection of moderate changes in TXA2 biosynthesis associated with 6-MP administration.

We also analyzed eicosanoid biosynthesis during myocardial ischemia (Figure 6). In patients randomized to 6-MP, LTE4 averaged 79±70 pg/mg creatinine (n=8) before 6-MP and 19±9 pg/mg creatinine (n=9) during glucocorticoid therapy (P<0.003). Similarly, 11-dehydro-TXB2 showed a significant (P<0.003) reduction from 341±323 to 51±27 pg/mg creatinine. In contrast, eicosanoid excretion did not change during placebo administration (67±50 versus 80±51 pg/mg creatinine and 722±782 versus 372±65 pg/mg creatinine, respectively; n=10 versus n=4).

**Effects of Myocardial Ischemia on Eicosanoid Biosynthesis in Stable Angina**

Eicosanoid excretion did not increase as a result of myocardial ischemia, as reflected by LTE4 and 11-dehydro-TXB2 values of 33±12 and 250±233 pg/mg creatinine (n=48), respectively, before the stress test and of 36±14 and 269±138 pg/mg creatinine (n=24) after effort-induced myocardial ischemia (Figure 7). Furthermore, the 6 patients who received 6-MP infusion immediately before the stress test did not show differences in either LTE4 (34±13 versus 36±16 pg/mg creatinine) or 11-dehydro-TXB2 (179±122 versus 273±285 pg/mg creatinine) in the samples collected before and after myocardial ischemia.

**Discussion**

The main finding of the present study is that in patients with unstable angina, enhanced LTC4 biosynthesis was significantly reduced by 6-MP with respect to placebo. Concurrent treatments or invasive procedures during the study are unlikely to have contributed to such a difference in LTC4 biosynthesis, inasmuch as these were fairly well balanced in the 2 groups (Table). The difference in LTE4 excretion

Figure 3. LTE4 excretion measured before and after randomization of unstable angina patients to IV 6-MP (top) or placebo (bottom). Dots represent individual measurements.

Figure 4. Effect of IV 6-MP (left) or placebo (right) on LTE4 excretion in patients with unstable angina. Dots depict data points from quadruple determinations made either before treatment or after treatment twice a day for 2 days.

Figure 5. The 11-dehydro-TxB2 excretion before (day 1) and after randomization of unstable angina patients to IV 6-MP or placebo. The bars and vertical lines represent mean±SD values.

Figure 6. The 11-dehydro-TxB2 excretion before (day 1) and after randomization of unstable angina patients to IV 6-MP or placebo. The bars and vertical lines represent mean±SD values.
became apparent after the first infusion of 6-MP and persisted throughout the second (P<0.005) and third (P<0.0001) day of study. Some alternative explanations might be considered for the mechanism(s) underlying the effect of 6-MP in suppressing cysteinyl LT biosynthesis during the acute phase of unstable angina. First, enhanced LTC4 biosynthesis might be merely a consequence of myocardial ischemia. Thus, when the severity of ischemia decreased as a function of time and therapy, it was associated with a lower LTE4 excretion. However, this seems unlikely inasmuch as this pattern was not observed in the unstable angina patients randomized to placebo. In addition, a similar decline in LTE4 during 6-MP administration was also found in the subset of samples collected during myocardial ischemia. Finally, in the patients with chronic stable angina, enhanced LTE4 excretion was uniformly distributed throughout the sampling period, both before and immediately after a positive exercise stress test. In agreement with our results, Takase et al reported that myocardial ischemia did not induce any significant changes in either arterial or coronary sinus plasma LTC4. Thus, the most plausible explanation is that the lower rate of LTC4 biosynthesis during 6-MP therapy may have reflected the inhibition by the drug of nucleated cellular sources of LTC4.

Cells in the vasculature, including polymorphonuclear leukocytes, platelets, and endothelial cells, are jointly involved in the biosynthesis of bioactive lipid mediators derived from arachidonic acid. Stimulation of human neutrophils alone resulted in production of LTA4, whereas only little production of LTC4 was observed. However, when erythrocyte, platelets, or endothelial cells were present in the incubation system, large amounts of LTC4 were produced from the neutrophil-derived LTA4. Because such transcellular metabolism may also occur in vivo and a persistent state of platelet and endothelial activation has been described in unstable angina, both endothelial cells and platelets, in the presence of neutrophils, could contribute to cysteinyl LT production in this setting. Glucocorticoids are able to reduce the expression of adhesion molecules on blood and vascular cell surfaces, an effect that could influence the platelet-leukocyte-endothelial cell interaction and could help explain the reduction in LTC4 biosynthesis observed in patients with unstable angina during 6-MP administration. Interestingly, these cell-cell interactions are aspirin-insensitive; therefore, aspirin-treated platelets, which are persistently activated in patients with unstable angina but not in those with stable angina, may be still capable of synthesizing the vasoconstrictor LTC4 from neutrophil LTA4 at a time when they can no longer produce TXA2. Thus, activated platelets, because of their quantity and reactivity, could contribute importantly to the generation of the biologically active LTC4 in unstable angina. Our findings indicate that a short-term regimen of 6-MP markedly reduced the in vivo

![Figure 6](http://circ.ahajournals.org/)

**Figure 6.** Urinary LTE4 and 11-dehydro-TXB2 measured during myocardial ischemia in unstable angina patients randomized to IV 6-MP (top) or placebo (bottom). The bars and vertical lines represent mean±SD values.

![Figure 7](http://circ.ahajournals.org/)

**Figure 7.** Eicosanoid metabolite excretion did not increase as a result of effort-induced myocardial ischemia in patients with stable angina. The bars, diamonds, and vertical lines represent mean±SD values.
LTC₄ biosynthesis in patients with acute unstable angina, whereas it was ineffective in patients with chronic stable angina. Because persistent platelet activation is generally observed in unstable angina but not in stable angina, areas of inflammatory infiltration are more frequently found and are larger in patients with acute coronary syndromes than in those with stable angina, as well as in those with symptomatic versus asymptomatic carotid plaques. Glucocorticoids can induce polymorphonuclear neutrophil reduction at the site of inflammation, thus our data are consistent with the hypothesis of an ongoing transcellular inflammatory process in the setting of coronary instability.

In this light, we cannot completely exclude the possibility that LTE₄ excretion in the 6-MP–treated group might be influenced by steroid-induced modifications in the number and/or localization of inflammatory cells. In fact, Santini et al recently described how in vivo administration of 6-MP in healthy subjects transiently increased the number of neutrophils by 90%, whereas it reduced the number of monocytes by 35%; this effect completely disappeared 24 hours after drug administration. Thus, the observation in the present study that neutrophil or monocyte counts were unchanged in blood samples collected 24, 48, and 72 hours after randomization in the patients taking 6-MP might be influenced by the time interval between blood collections.

The molecular mechanism(s) of glucocorticoid action on LTC₄ biosynthesis is still unknown. Glucocorticoids can act at different levels of gene regulation, depending on cell type and inducing stimulus. Evidence from studies in human subjects and animal models suggests that the anti-inflammatory effect might occur through induction of the synthesis of a family of proteins (annexins, also known as lipocortins) that have a pivotal role in modulating inflammatory cell activation, adhesion molecule expression, and migratory and phagocytic functions and that also interfere with the early step of arachidonate metabolism by inhibiting phospholipase A₂. Furthermore, glucocorticoids may also inhibit phospholipase A₂ activation and arachidonic acid release through a glucocorticoid receptor–dependent, transcription-independent mechanism. Alternatively, glucocorticoids may reduce inflammation by inducing cell apoptosis via an autocrine or paracrine pathway involving the up-regulation of the death receptor CD95 and its ligand CD95L on cell membranes.

In our in vivo study, 6-MP failed to influence overall 11-dehydro-TXB₂ excretion, despite our recent ex vivo evidence that single oral doses of 6-MP caused a time-dependent inhibition of whole-blood COX-2 activity in healthy subjects. However, in contrast to in vitro and ex vivo studies, no specific markers of COX-2 activity in circulating inflammatory cells are available for in vivo studies; therefore, the interpretation of this discrepancy remains open to different possibilities. The most plausible explanation is that the small sample size and large interindividual variability in 11-dehydro-TXB₂ excretion in the present study would preclude detection of moderate changes in TXA₂ biosynthesis associated with 6-MP administration. Alternatively, because increased COX-1 expression in monocytes occurs after lipopolysaccharide injection in humans, synergistic induction of COX-1 in nucleated cells exposed to inflammatory cytokines could represent an unusual mechanism for TXA₂ biosynthesis in aspirin-treated patients with unstable angina during glucocorticoid therapy. Finally, because glucocorticoids were able to inhibit TXA₂ biosynthesis in the samples collected during myocardial ischemia but not in those collected during the ischemia-free periods, we cannot exclude the possibility that different cellular sources with diverse sensitivity to glucocorticoids may be active in the various phases of acute unstable angina.

Finally, the present study also shows that permanent inactivation of platelet COX activity by low-dose aspirin and subsequent increased availability of arachidonic acid as substrate is not associated with altered biosynthesis of LTC₄ in healthy subjects or in patients with coronary artery disease.

We did not perform any measurements of 5-lipoxygenase and COX-2 activity in the circulating cells of our patients; therefore, the interpretation of our findings remains open to different possibilities. Despite this limitation, we think that our findings have both research and clinical implications. LTE₄ excretion in aspirin-treated unstable angina patients most likely reflects the involvement of glucocorticoid-sensitive inflammatory cells and may represent a useful biochemical end point for dose-finding studies of anti-inflammatory interventions in this setting. Moreover, aspirin–insensitive eicosanoid biosynthesis might provide a mechanism for the episodic formation of potent mediators of vascular smooth muscle cell contraction and platelet activation, possibly contributing to clinical ischemic events. The availability of 6-MP, as well as LT receptor antagonists and inhibitors of LT biosynthesis, now offers the opportunity of testing this hypothesis. Whether this hypothesis will be confirmed in larger clinical trials is unknown; if so, case-selective inhibition of the LT pathway should be considered a potential therapeutic strategy in patients with unstable angina.

Acknowledgments

We are indebted to Carlo Patrono for helpful suggestions in the design of the study and preparation of the manuscript.

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_Circulation_. 2003;107:55-61; originally published online December 2, 2002; doi: 10.1161/01.CIR.0000043260.82447.62
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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