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Thrombopoietin Contributes to Enhanced Platelet Activation in Patients With Unstable Angina

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OBJECTIVES We sought to investigate the potential role of elevated levels of thrombopoietin (TPO) in platelet activation during unstable angina (UA).

BACKGROUND Thrombopoietin is a humoral growth factor that does not induce platelet aggregation per se, but primes platelet activation in response to several agonists. No data concerning its contribution to platelet function abnormalities described in patients with UA are available.

METHODS We studied 15 patients with UA and, as controls, 15 patients with stable angina (SA) and 15 healthy subjects. We measured TPO and C-reactive protein (CRP), as well as monocyte-platelet binding and the platelet expression of P-selectin and of the TPO receptor, c-Mpl. The priming activity of patient or control plasma on platelet aggregation and monocyte-platelet binding and the role of TPO in this effect also were studied.

RESULTS Patients with UA showed higher circulating TPO levels, as well as increased monocyte-platelet binding, platelet P-selectin expression, and CRP levels, than those with SA and healthy control subjects. The UA patients also showed reduced platelet expression of the TPO receptor, c-Mpl. In vitro, the plasma from UA patients, but not from SA patients or healthy controls, primed platelet aggregation and monocyte-platelet binding, which were both reduced when an inhibitor of TPO was used.

CONCLUSIONS Thrombopoietin may enhance platelet activation in the early phases of UA, potentially participating in the pathogenesis of acute coronary syndromes. (J Am Coll Cardiol 2006;48: 2195–203) © 2006 by the American College of Cardiology Foundation

Patients with unstable angina (UA) have hypersensitive platelets, circulating platelet aggregates, and elevated levels of circulating platelet secretory products (1–5). They also show increased platelet-leukocyte aggregates, which have been proposed to represent a better reflection of plaque instability and ongoing vascular thrombosis and inflammation (6,7). Indeed, activated platelets deposit at sites of unstable plaque rupture and may potentiate thrombus formation, precipitating or exacerbating coronary vascular obstruction (3). The clinical efficacy of antiplatelet therapies also confirms the importance of platelets in acute coronary syndromes (8); however, their incomplete effectiveness suggests alternative platelet activation pathways may be important (8).

Thrombopoietin (TPO) is a humoral growth factor originally identified using antisense oligonucleotides to c-Mpl (9), a protooncogene described by Wendling et al. (10) in 1986, and characterized for its ability to stimulate the proliferation and differentiation of megakaryocytes (11–14). Thrombopoietin is constitutively produced by the liver and kidneys and is then cleared from circulation upon binding with its receptor, c-Mpl, expressed mainly on platelets and megakaryocytes (15,16). Elevated plasma TPO levels have been reported in different clinical conditions, including hematologic diseases, where increased circulating TPO may be a response to altered bone marrow hemopoiesis (17–19) and sepsis (20). Moreover, higher levels of TPO have been found in patients with acute coronary syndromes than in control subjects and shown to correlate with platelet size, thus potentially resulting in hemostatically more active platelets (21). We and others have shown that TPO directly modulates the response of mature platelets to several stimuli and thereby their homeostatic potential (22,23). In particular, TPO, which does not induce platelet aggregation per se, enhances platelet activation in response to several agonists (22,23) and the subsequent platelet/leukocyte adhesion via P-selectin (24).

The aim of the present study was to investigate the potential pathogenic role of elevated levels of TPO in sustaining platelet aggregation and monocyte-platelet interaction in UA patients. We measured TPO levels in patients with unstable and stable angina (SA) and healthy controls and found that patients with UA showed higher circulating TPO levels than those with SA and healthy controls, as well as increased markers of platelet activation in vivo. In addition, we showed that plasma from patients with UA, but not from patients with SA or healthy controls, primes platelet activation in vitro and that TPO has a role in this effect.

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JACC Vol. 48, No. 11, 2006
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Abbreviations and Acronyms

ADP = adenosine 5′-diphosphate
CRP = C-reactive protein
EPI = epinephrine
PI = priming index
PRP = platelet-rich plasma
rhTPO = recombinant human thrombopoietin
SA = stable angina
TPO = thrombopoietin
TPOR = thrombopoietin receptor
UA = unstable angina

METHODS

Patients. The study group comprised 15 patients with UA who had ischemic chest pain at rest with transient ST-segment depression and/or T-wave inversion (Braunwald class IIIB [25]) within the preceding 8 h, 15 SA patients, and 15 subjects without clinical evidence of coronary atherosclerosis used for controls. The SA patients had stable effort angina (Canadian Cardiovascular Society class II to III), a positive exercise test, and angiographically documented coronary atherosclerotic lesions. The UA patients were enrolled at the time of hospital admission, and blood sampling was performed in the emergency room before any therapeutic intervention was started. Exclusion criteria were advanced kidney or liver failure, overt heart failure, known cardiomyopathy or hemodynamically significant valvular heart disease, known hematologic disease affecting coagulation, platelet, or TPO production (16–18,26), known malignancies, inflammatory diseases, and major surgery (27) or trauma within the preceding month. We considered patients who were receiving dietary treatment or medication for diabetes or whose fasting blood glucose level was above 126 mg/dl twice to have diabetes mellitus. Angiographically severe coronary artery disease was defined by the presence of 1 or more stenoses of at least 70% in any major coronary artery. The study was approved by the hospital ethics committee and conducted according to the Helsinki Declaration. All subjects gave informed consent.

Clean venipunctures were performed by trained investigators using 19-gauge butterfly infusion sets without venous stasis. After discarding the first 4 ml, blood entered Vacutainers (Becton Dickinson, Franklin Lakes, New Jersey) containing EDTA or 3.8% trisodium citrate as appropriate. To obtain plasma samples, EDTA-anticoagulated tubes were centrifuged at 1,600 *g* for 10 min at 4°C. Plasma was then centrifuged again at 12,500 *g* for 10 min at 4°C, filtered through 0.22 μm pores, and immediately frozen and stored at −70°C.

Biochemical analyses. The TPO levels in plasma were measured using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota) following the manufacturer's instructions. C-reactive protein (CRP) was assayed by high-sensitivity nephelometry (Dade Behring, Marburg, Germany).

Flow cytometry. Leucocyte-platelet aggregates in vivo were analyzed by 3-color staining of whole blood samples, as previously described (28). Briefly, blood was collected, diluted 1:1 with Tyrodes' HEPES-buffered saline (pH 7.4), and added to a mixture of fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (Beckman Coulter, Miami, Florida), energy coupled dye-conjugated anti-CD14 (Beckman Coulter, Marseille, France), and phycoerythrin-conjugated anti-CD41 (Dako Cytomation, Glostrup, Denmark) monoclonal antibodies, and incubated 15 min at room temperature. Cells were then fixed with 1% paraformaldehyde, and erythrocytes were removed by hypotonic lysis. Samples were washed twice with phosphate-buffered saline (PBS)–bovine serum albumin 0.1% and resuspended in 0.5 ml PBS.

Samples were analyzed on an EPICS-XL flow cytometer (Coulter, Hialeah, Florida). Gates were set during analysis on the ground of CD45 versus CD14 expression using a logical gate to discriminate lymphocyte, polymorphonuclear, and monocyte populations. The percentage of leukocyte subgroups coexpressing CD45-CD41 or CD14-CD41 over the total population of leukocytes expressing CD45 or CD14 was used as an index of platelet-leukocyte adhesion (29).

For in vitro experiments, 100 μl diluted blood from healthy adult donors was preincubated at 37°C with 25 μl plasma from patients or control subjects or recombinant human TPO (rhTPO) (R&D Systems) for 5 min and then stimulated with adenosine 5′-diphosphate (ADP) (0.8 μmol/l; Helena Laboratories, Beaumont, Texas) or epinephrine (EPI) (3 μmol/l; Helena Laboratories). Samples were then processed and analyzed as described in the preceding text.

In separate experiments, plasma or rhTPO was incubated with a human TPO receptor (TPOR)–Fc chimera (2.5 μg/ml; R&D Systems) for 5 min at 37°C; the mixture of sample and TPOR-Fc chimera was added to whole blood, further incubated for 5 min at 37°C, and stimulated with ADP or EPI.

P-selectin expression was evaluated in whole blood using an FITC-conjugated anti–P-selectin monoclonal antibody from Ancell Corporation (Bayport, Minnesota). Platelet c-Mpl surface expression was examined in fixed platelet-rich plasma (PRP) using an anti–c-Mpl monoclonal antibody (R&D Systems) followed by Alexa Fluor 488–conjugated antimouse immunoglobulin G monoclonal antibody (Molecular Probes, Eugene, Oregon).

Platelet aggregation. Platelet aggregation in PRP and whole blood was evaluated as previously described (22,28). Platelet-rich plasma or whole blood was obtained from healthy subjects as described in the preceding and incubated with 25 to 100 μl plasma or rhTPO at 37°C. When evaluating priming activity, ADP or EPI was added as secondary agonist. For each experiment, the ADP or EPI concentration that induced the minimum measurable aggregation was determined; ADP dose range was 0.8 to 2

Table 1. Characteristics of the Patients*

Characteristics	Healthy Controls (n = 15)	SA Patients (n = 15)	UA Patients (n = 15)
Age, yrs	66.3 ± 3.4	65.5 ± 3.4	68.0 ± 2.2
Gender, n			
Male	9	12	10
Female	6	3	5
Risk factors, n (%)			
Family history	3 (20.0)	5 (33.3)	5 (33.3)
Hypertension	5 (33.3)†	13 (86.7)	14 (93.3)
Current smoking	4 (26.7)	3 (20.0)	1 (6.7)
Hypercholesterolemia	2 (13.3)†	15 (100.0)	14 (93.3)
Diabetes	0†	5 (33.3)	5 (33.3)
Therapy (at the time of hospital presentation), n (%)			
Anti-platelet			
ASA	0†	13 (86.7)	15 (100.0)
Ticlopidine	0	2 (13.3)	1 (6.7)
Clopidogrel	0	1 (6.7)	1 (6.7)
Beta-blockers	2 (13.3)†	10 (66.7)	12 (80.0)
ACE inhibitors/ARBs	5 (33.3)	6 (40.0)	8 (53.3)
Nitrates	0†	8 (53.3)	10 (66.7)
Statins	0†	8 (53.3)	10 (66.7)
Calcium-channel blockers	0†	5 (33.3)	4 (26.7)
Angiographic findings			
1-vessel disease	n.d.	1 (6.7)	1 (6.7)
2-vessel disease	n.d.	5 (33.3)	4 (26.7)
3-vessel disease	n.d.	9 (60.0)	10 (66.7)
Platelets (10^9/l)	200.8 ± 11.9	202.1 ± 13.2	205.5 ± 14.1
MPV (fl)	10.8 ± 0.2	10.9 ± 0.4	11.7 ± 0.4
WBC (10^6/l)	6,605.4 ± 706.0	6,808.0 ± 688.4	6,397.1 ± 254.4
Monocytes (10^6/l)	400.1 ± 35.8	358.0 ± 52.4	426.0 ± 67.8

*Plus-minus values are mean ± SE. †$p < 0.05$ for the comparison with the other 2 groups.
ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; ASA = acetylsalicylic acid; MPV = mean platelet volume; n.d. = not determined; SA = stable angina; UA = unstable angina; WBC = white blood cells.

μmol/l; EPI dose range was 0.1 to 3 μmol/l. The priming index (PI) was calculated as the response to plasma or rhTPO and agonist together, divided by the sum of individual responses elicited by plasma or rhTPO and agonist (22,28).

In separate experiments, plasma or rhTPO was incubated with the TPOR-Fc chimera (2.5 μg/ml) for 5 min at 37°C; the mixture of sample and TPOR-Fc chimera was added to PRP or whole blood, further incubated for 5 min at 37°C, and stimulated with ADP or EPI.

In selected experiments, PRP was preincubated with acetylsalicylic acid (0.1 mmol/l; Sigma Chemical Co., St. Louis, Missouri) for 30 min at 37°C before stimulation with plasma or rhTPO, followed by ADP or EPI.

Tyrosine phosphorylation of c-Mpl. In some experiments, PRP was prepared from patients or controls, stimulated with rhTPO (100 ng/ml) for 10 min, and then lysed in lysis buffer (20 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1% Nonidet P-40) containing protease and phosphatase inhibitors. Immunoprecipitation was performed using a rabbit polyclonal antibody anti–c-Mpl (Upstate Biotechnology, Lake Placid, New York). After SDS-PAGE, immunoprecipitated proteins were transfered to nitrocellulose membranes and blotted with anti-phosphotyrosine monoclonal antibody PY20 (Sigma Chemical Co.).

Statistical analysis. Values are represented by mean ± SE. Comparisons between groups were analyzed by 1-way analysis of variance followed by Newman-Keuls multicomparison test; categoric variables were compared by 2-way cross-tabulation with the chi-square test. A p value of <0.05 was considered significant.

RESULTS

Patient clinical characteristics and TPO levels. Table 1 gives demographic and clinical data for patients and controls. The UA and SA patients did not differ regarding demographic and baseline clinical characteristics or treatment. Platelet, leukocyte, and monocyte counts were not significantly different in the 3 groups and neither was mean platelet volume. In contrast, plasma TPO concentrations were significantly higher in patients with UA than in those with SA or in healthy control subjects (Fig. 1A). The CRP values were also significantly higher in UA (5.23 ± 1.31 mg/l) than in SA (2.19 ± 0.41 mg/l) patients or in healthy control subjects (1.83 ± 0.54 mg/l).

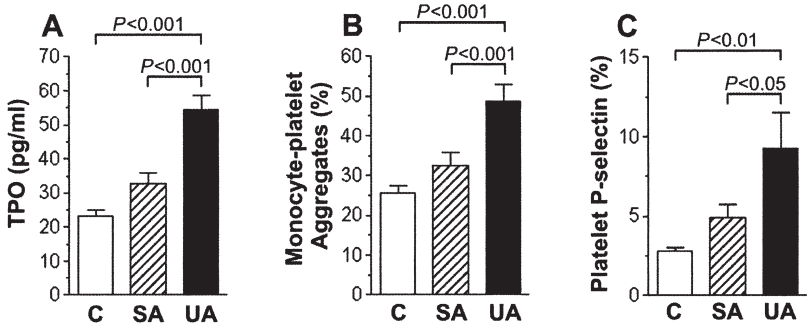

Figure 1. **(A)** Plasma thrombopoietin (TPO) levels in healthy control subjects (C) and in patients with stable angina (SA) and unstable angina (UA). **(B)** Monocyte-platelet aggregates and **(C)** platelet P-selectin expression detected in vivo, by flow cytometry, in healthy control subjects and in patients with SA and UA. One-way analysis of variance with Newman-Keuls multicomparison test was performed.

In vivo platelet activation in patients with UA. Significantly higher percentages of monocyte-platelet binding were found in unstable than in SA patients or healthy controls (Fig. 1B). The percentage of platelets expressing P-selectin was also significantly higher in unstable than in SA patients or healthy controls (Fig. 1C).

Decreased platelet expression of c-Mpl in UA patients. Because c-Mpl expression is down-regulated following internalization upon TPO binding (30,31), we examined platelet c-Mpl expression as an indirect marker of in vivo platelet activation by circulating endogenous TPO. The c-Mpl expression was markedly lower in UA than in SA patients or in healthy control subjects (Figs. 2A and 2B).

When we examined the phosphorylation state of c-Mpl receptor in platelets stimulated in vitro with saturating rhTPO levels, we found that c-Mpl was phosphorylated in platelets from SA patients and healthy control subjects but not in platelets from UA patients, showing that in UA patients the receptor was functionally non-responding to TPO stimulation in vitro (Fig. 2C).

Finally, rhTPO did not enhance the aggregation induced by ADP or EPI in PRP from patients with UA, whereas it

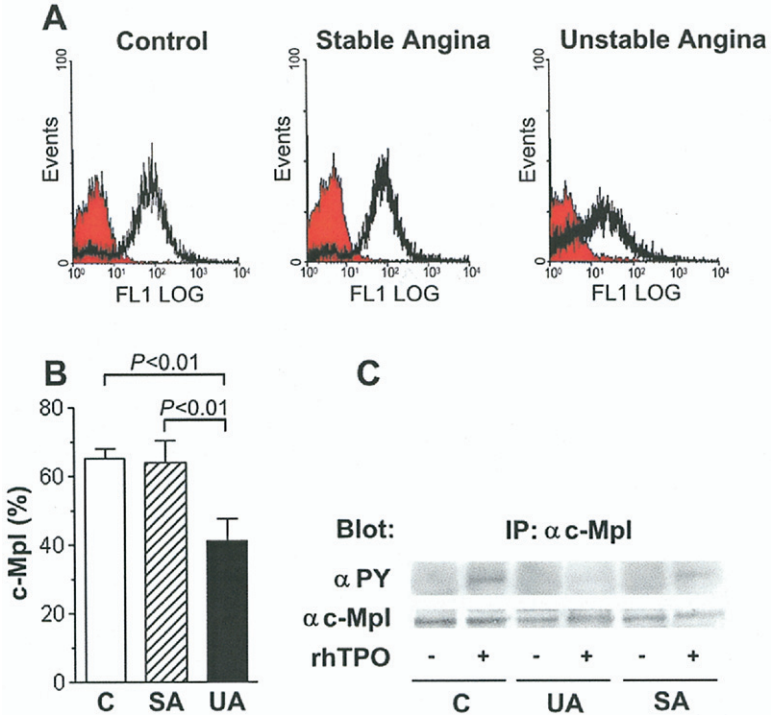

Figure 2. **(A)** Representative fluorescence-activated cell sorting (FACS) analysis showing the percentage of c-Mpl expression on platelet surface detected in vivo in healthy control subjects (C) and in patients with stable angina (SA) and unstable angina (UA). The **solid curve** represents fluorescence due to binding of non-specific antimouse immunoglobulin G monoclonal antibody; the **open curve** represents fluorescence due to binding of anti–c-Mpl monoclonal antibody. **(B)** Quantification of c-Mpl expression on platelet surface detected in vivo in healthy controls and in patients with SA and UA by FACS analysis. One-way analysis of variance with Newman-Keuls multicomparison test was performed. **(C)** Representative Western blot analysis of the phosphorylation state of c-Mpl in platelet lysates from healthy control subjects and from patients with SA and UA. Platelets from control subjects or patients were either unstimulated or stimulated with saturating concentrations of recombinant human thrombopoietin (rhTPO). The c-Mpl was phosphorylated in platelets from SA patients and healthy control subjects but not in platelets from UA patients. Shown is a representative experiment out of 4 with similar results.

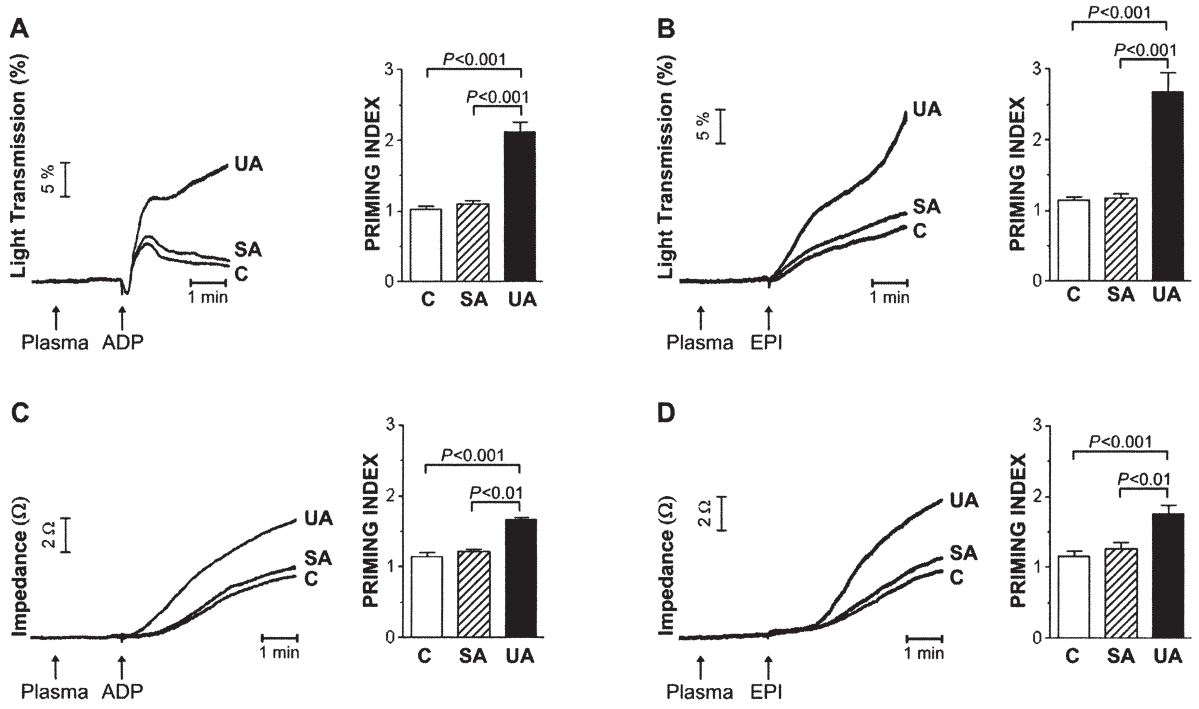

Figure 3. Representative aggregation traces and quantification of the in vitro priming activity induced by plasma from healthy control subjects (C) and patients with stable angina (SA) and unstable angina (UA) on **(A)** adenosine 5′-diphosphate (ADP)- or **(B)** epinephrine (EPI)-induced platelet aggregation in PRP. Representative aggregation traces and quantification of the in vitro priming activity induced by plasma from healthy controls and patients with SA and UA on **(C)** ADP- or **(D)** EPI-induced platelet aggregation in whole blood. One-way analysis of variance with Newman-Keuls multicomparison test was performed.

primed platelet aggregation in PRP from patients with SA and from healthy control subjects (data not shown). This suggests that increased TPO levels in vivo may account for the reduced reactivity to rhTPO in vitro in PRP from patients with UA.

Plasma of UA patients primed platelet activation in vitro. The effect of plasma samples from patients or controls on platelet aggregation was tested in vitro on PRP or whole blood obtained from healthy donors. Plasma from UA patients per se did not induce platelet aggregation, but it significantly enhanced aggregation induced by low ADP or EPI concentrations in PRP (Figs. 3A and 3B). All plasma samples examined induced this priming effect on platelet aggregation in PRP. Also in whole blood, preincubation of samples with plasma from UA patients primed aggregation induced by ADP or EPI (Figs. 3C and 3D). The priming effect of plasma samples from UA patients was evident after 5 min of incubation with PRP or whole blood and persisted up to 60 min. Moreover, it was still significant after pretreatment of PRP with acetylsalicylic acid (0.1 mmol/l), which partially reduced ADP-induced platelet aggregation (from 2.40 ± 0.50 to 1.77 ± 0.31) and inhibited the secondary phase of EPI-induced platelet aggregation (from 2.30 ± 0.02 to 1.72 ± 0.04). Finally, plasma of UA patients also induced a significant, although weaker, priming effect in PRP from healthy subjects after 1 week of oral treatment with acetylsalicylic acid (100 mg/day) (data not shown). Plasma from SA patients or healthy control subjects did not prime platelet aggregation in PRP nor in whole blood (Fig. 3).

In parallel experiments, we used rhTPO to investigate the dose range needed to induce a priming effect on platelet aggregation. Alone, rhTPO (from 20 pg/ml to 80 ng/ml) did not induce platelet aggregation in PRP nor in whole blood but enhanced platelet aggregation when administered before ADP or EPI. The minimal "priming" dose of rhTPO was 20 pg/ml in PRP and 50 pg/ml in whole blood. These concentrations are similar to those detected in vivo in the circulation of UA patients. No priming effect on ADP- or EPI-induced platelet aggregation was seen using interleukin-6, interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor (data not shown).

Plasma from UA patients, but not from SA patients or healthy control subjects, also significantly enhanced monocyte-platelet binding and P-selectin expression in whole blood (Fig. 4).

Role of TPO in the priming activity of plasma from UA patients. We hypothesized that the soluble plasmatic factor responsible for the effect of plasma from patients with UA on platelet aggregation and monocyte-platelet binding would be TPO. To test this hypothesis, the contribution of TPO to platelet activation was assessed by using a human TPOR-Fc chimera, which inhibits the proliferation induced by rhTPO on MO7e cells (32). In preliminary experiments, we found that the TPOR-Fc chimera, at a concentration of

JACC Vol. 48, No. 11, 2006
December 5, 2006:2195–203

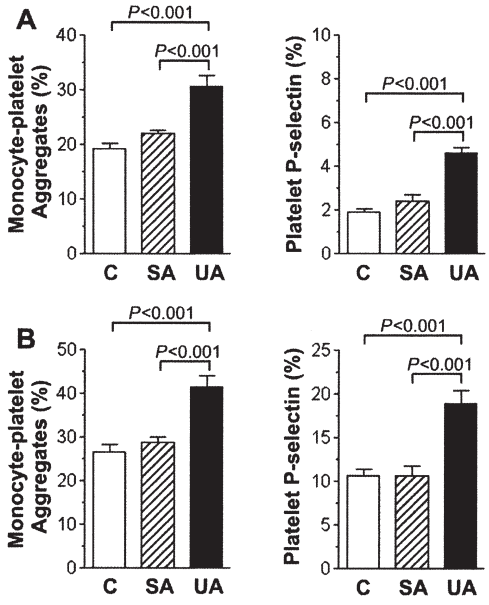

Figure 4. **(A)** In vitro effect of plasma from healthy control subjects (C) and patients with stable angina (SA) and unstable angina (UA) on adenosine 5′-diphosphate-induced monocyte-platelet aggregation **(left)** and platelet P-selectin expression **(right)** in whole blood. **(B)** In vitro effect of plasma from healthy control subjects and patients with SA and UA on epinephrine-induced monocyte-platelet aggregation **(left)** and platelet P-selectin expression **(right)** in whole blood. Monocyte-platelet aggregates and platelet P-selectin expression were analyzed by flow cytometry. Analysis of variance with Newman-Keuls multicomparison test was performed.

2.5 μg/ml, inhibited the priming effect exerted by rhTPO in PRP but did not affect the aggregation induced by ADP or EPI. Pretreatment of plasma sample with the TPOR-Fc chimera reduced by about 80% the priming effect exerted by plasma from UA patients on platelet aggregation in both PRP (Figs. 5A and 5B) and whole blood (Figs. 5C and 5D). It also significantly decreased monocyte-platelet binding and P-selectin expression observed after preincubation of the sample with plasma from UA patients (Fig. 6). In contrast, preincubation of plasma from SA patients or healthy control subjects with the TPOR-Fc chimera had no effect (data not shown).

Finally, we adjusted the concentrations of TPO in plasma from SA patients to those measured in UA patients by adding exogenous rhTPO. After addition of rhTPO, plasma from SA patients, which did not show priming activity per se, was able to induce a significant priming effect, similar to that observed with plasma from patients with UA, on ADP- or EPI-induced aggregation in PRP (PI = 1.89 ± 0.15 on ADP-induced aggregation, $p < 0.01$; 2.45 ± 0.34 on EPI-induced aggregation, $p < 0.05$).

DISCUSSION

The present study demonstrates the importance of TPO in the pathophysiology of increased platelet activation during UA. We showed indeed that patients with UA have high circulating TPO levels together with increased monocyte-platelet binding and platelet P-selectin expres-

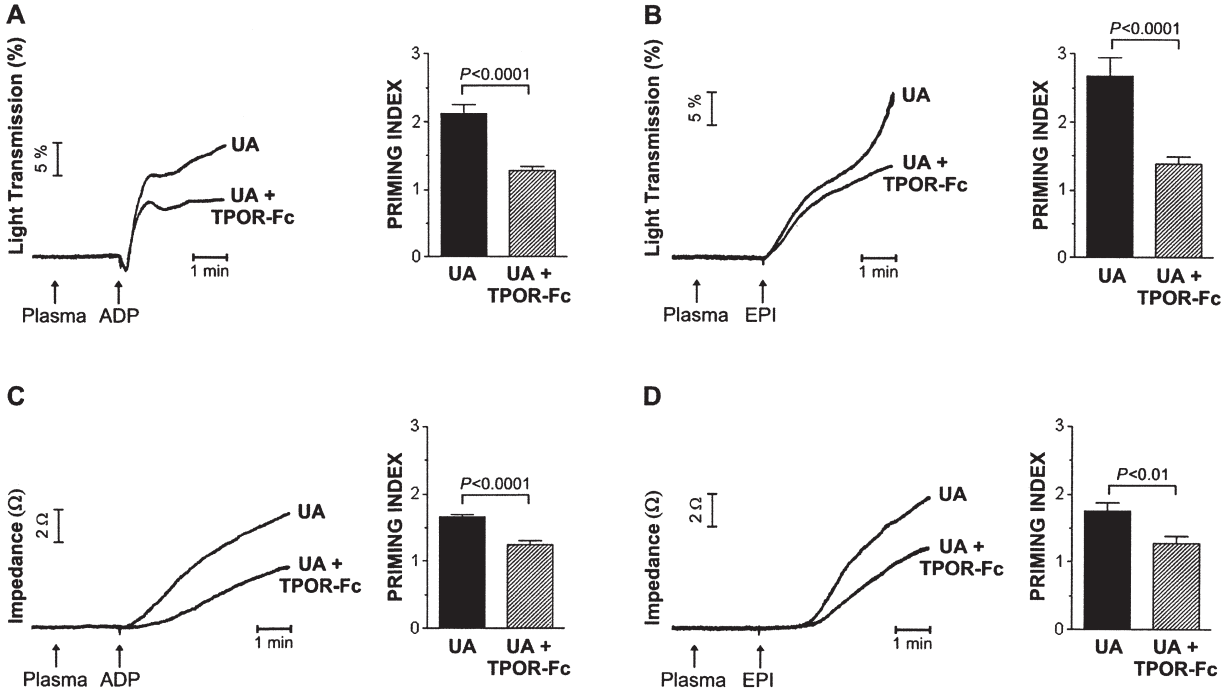

Figure 5. Representative aggregation traces and quantification of the effect of thrombopoietin receptor (TPOR)-Fc chimera on the in vitro priming activity induced by plasma from patients with unstable angina (UA) on **(A)** adenosine 5′-diphosphate (ADP)- or **(B)** epinephrine (EPI)-induced platelet aggregation in platelet-rich plasma. Representative aggregation traces and quantification of the effect of TPOR-Fc chimera on the in vitro priming activity induced by plasma from UA patients on **(C)** ADP- or **(D)** EPI-induced platelet aggregation in whole blood. One-way analysis of variance with Newman-Keuls multicomparison test was performed.

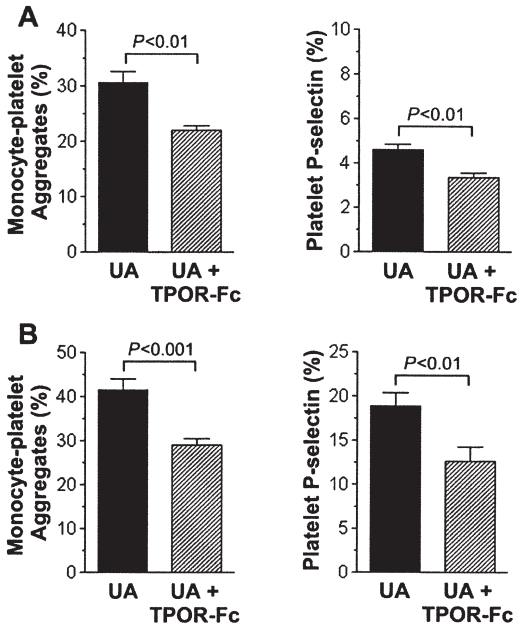

Figure 6. (A) Effect of thrombopoietin receptor (TPOR)-Fc chimera on the priming activity induced by plasma from patients with unstable angina (UA) on adenosine 5′-diphosphate (ADP)-induced monocyte-platelet aggregation **(left)** and platelet P-selectin expression **(right)** in whole blood. **(B)** Effect of TPOR-Fc chimera on the priming activity induced by plasma from patients with UA on epinephrine (EPI)-induced monocyte-platelet aggregation **(left)** and platelet P-selectin expression **(right)** in whole blood. Pretreatment of plasma sample with the TPOR-Fc chimera significantly reduced the percentages of ADP- or EPI-induced monocyte-platelet binding and platelet P-selectin expression observed after preincubation of whole blood from healthy adult donors with plasma from UA patients. Analysis of variance with Newman-Keuls multicomparison test was performed.

sion in vivo, and that TPO present in the plasma of patients with UA may have a pathogenic role in this condition by priming platelet aggregation and monocyte-platelet interaction.

Confirming what was previously reported by Senaran et al. (21), we found higher circulating TPO levels in patients with UA, at the time of hospital admission, than in patients with SA or in healthy control subjects. However, whereas that earlier study was conducted within 12 h after hospital admission (21) (i.e., when several clinical factors and therapeutic interventions may have already intervened), we enrolled the UA patients in the emergency room before any therapeutic intervention was started. Therefore, we believe that our data may more closely reflect the ongoing pathogenic events leading to the development of UA.

Patients with UA also showed reduced surface expression of c-Mpl on circulating platelets, whereas no such a change was detected in patients with SA or healthy controls. Receptor-mediated internalization is considered the primary means of regulating plasma TPO levels (30,31). Diminished c-Mpl expression in platelets from UA patients may therefore depend on the previous binding of TPO to its receptor in vivo, followed by its internalization and surface down-regulation (30,31). In addition, rhTPO is able to phosphorylate c-Mpl only in platelets from SA patients and healthy control subjects but not in those from UA patients, further supporting this hypothesis. Platelets from patients with UA also showed reduced reactivity to rhTPO ex vivo, possibly related to their exposure in vivo to increased levels of endogenous TPO, analogously to what was previously shown for thrombocytopenic mice (33) and humans with the syndrome of congenital thrombocytopenia with absent radii (33,34).

In addition to elevated plasmatic concentrations of TPO, patients with UA also showed increased indexes of in vivo platelet activation, such as monocyte-platelet binding and platelet P-selectin expression. It is well known that platelet activation occurs during the early phases of UA (1–6,8) and that monocyte-platelet aggregation is not only a sensitive measure of platelet activation but also has significant proinflammatory consequences (6,7,35,36). Thus, our in vivo and in vitro findings suggest an important link between circulating TPO level and the proinflammatory and prothrombotic state that occurs in UA patients.

The precise origin of the rise in plasma TPO level in UA patients remains unclear. We found that CRP levels were increased in this study group, suggesting that the liver acute-phase response, which takes place in acute coronary syndromes (37–39), may have a role in increasing TPO levels. Considering the evidence that elevated CRP has independent prognostic value in UA (40,41), it is tempting to speculate that the negative prognostic implications of high CRP levels in patients with UA may be at least partially related to the concomitant increase in TPO production and subsequent priming of platelet aggregation. However, activated platelets could also represent a major contributor to the elevated TPO levels observed in UA patients, because they are known to release full-length biologically active TPO upon stimulation (26).

In vitro, plasma from patients with UA, but not from SA patients or healthy control subjects, markedly enhances platelet aggregation as well as monocyte-platelet binding in blood samples from healthy donors, stressing the importance of elevated TPO concentrations in the pathogenesis of increased platelet aggregation in UA. Several aspects of the data show that the priming effect exerted by plasma samples from patients with UA may be due to their content in TPO: 1) the priming effect induced by plasma from UA patients was significantly decreased when TPO activity was inhibited with a TPOR-Fc chimera; and 2) adjusting the concentrations of TPO in plasma from SA patients to those measured in UA patients by adding exogenous rhTPO induced a significant increase of the priming effect, similar to that observed with plasma from patients with UA, on ADP- or EPI-induced aggregation in PRP. In vivo, a similar priming effect induced by TPO on platelet activation has been documented in non-human primates; platelets derived from TPO-treated animals showed a heightened sensitivity to substances that stimulate platelet aggregation during the first few days of treatment (42). No priming effect on ADP- and EPI-induced platelet aggregation in both PRP and

whole blood was seen using interleukin-6, interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor; however, other soluble mediators released during UA have been shown to affect platelet function (43,44).

Plasma from UA patients also induced a significant priming effect on platelet aggregation in the presence of acetylsalicylic acid or in PRP from healthy subjects after 1 week of oral acetylsalicylic acid treatment. These results suggest that the activation pathway triggered by TPO is only partially affected by the anti-platelet therapy commonly used in patients with myocardial ischemia (8) and that platelet priming by TPO may represent a mechanism leading to therapeutic failure of anti-platelet agents. Moreover, the phenomenon we described in this study may provide the rationale for more aggressive (double or triple) anti-platelet treatment in patients with acute coronary syndromes.

In conclusion, we have shown that elevated levels of circulating TPO may enhance platelet activation and monocyte-platelet interaction in the early phases of UA. These findings implicate TPO in the pathogenesis of acute coronary syndromes, where it could potentially precipitate conditions of clinical instability.

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