Thrombopoietin, c-Mpl ligand, induces tyrosine phosphorylation of Tyk2, JAK2, and STAT3, and enhances agonists-induced aggregation in platelets in vitro

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Abstract We investigated in vitro effects of recombinant human thrombopoietin (TPO), or c-Mpl ligand, on human platelets. TPO induced rapid dose-dependent tyrosine phosphorylation of several proteins. We identified Janus tyrosine kinases, Tyk2 and JAK2, and a member of STAT (signal transducers and activators of transcription) family, STAT3, as the tyrosine-phosphorylated proteins in response to TPO. TPO by itself did not cause platelet aggregation and shape change, but augmented ADP-induced aggregation in a dose-dependent manner. Acetylsalicylic acid inhibited the secondary aggregation enhanced by TPO, but not the TPO-induced potentiation of the primary aggregation. TPO modulates platelet activation possibly through protein-tyrosine phosphorylation.

Key words: Thrombopoietin; Signal transduction; Protein-tyrosine kinase; Transcription factor; Platelet aggregation; Adenosine diphosphate

1. Introduction

Many previous studies have suggested that lineage-specific humoral factors regulate platelet production [1]. Recently, several groups [2–5] cloned the ligand for the c-Mpl proto-oncogene [6] which is a member of the cytokine receptor superfamily. These studies and others have demonstrated that c-Mpl ligand plays a critical role in megakaryocytopoiesis and thrombopoiesis both in vitro and in vivo [2–5,7–10]. Therefore, c-Mpl ligand has been termed thrombopoietin (TPO) [3,7,8] or megakaryocyte growth and development factor (MGDF) [4]. The cytokine receptor superfamily, which lacks a tyrosine kinase domain, couples ligand binding with the induction of protein-tyrosine phosphorylation that is essential for cytokine signaling [11]. The Janus tyrosine kinase (JAK) family, JAK1, JAK2, JAK3, and Tyk2, and the STAT (for signal transducers and activators of transcription) family are activated through phosphorylation on tyrosine in response to cytokines and growth factors [12,13]. JAKs and STATs provide novel common pathways shared by the receptors for various cytokines or growth factors. Some researchers [14–17] have reported that TPO stimulates tyrosine phosphorylation of molecules including JAK2 and STATs in megakaryocytic cell lines or cells engineered to express the c-Mpl.

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Abbreviations: TPO, thrombopoietin; JAK, Janus tyrosine kinase; ASA, acetylsalicylic acid or aspirin; PRP, platelet-rich plasma; STAT, signal transducers and activators of transcription.

A very recent report has shown that c-Mpl is expressed in megakaryocytic lineage from late progenitors to platelets [18]. Another study showed that TPO enhances ADP-induced platelet aggregation and that this activation is blocked by acetylsalicylic acid, aspirin, (ASA) [19]. In the present study we investigated in vitro effect of recombinant human TPO on human platelets. Our findings indicated that TPO stimulated tyrosine phosphorylation of several proteins including the JAK and STAT families, and possessed ASA-insensitive potentiation effect on platelet aggregation.

2. Materials and methods

2.1. Reagents

Recombinant human TPO was a kind gift from Kirin Brewery Co. (Maebashi, Japan). Anti-phosphotyrosine mAb 4G10 and rabbit anti-STAT2 polyclonal antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Affinity-purified rabbit polyclonal antibodies against JAK1 (HR-785), JAK2 (HR-758), JAK3 (C-21), Tyk2 (C-20), STAT3 (C-20), and STAT4 (C-20) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-STAT1α/β mAb (C-136) recognizes both STAT1α and -1β, and was obtained from Santa Cruz Biotechnology. Anti-STAT5 and anti-STAT6 mAbs were from Transduction Laboratories (Lexington, KY). Affinity-purified rabbit anti-mouse IgG was obtained from Organon Teknika Corp. (Durham, NC). PGE_1 was kindly provided by Ono Pharmaceutical Co. (Osaka, Japan). Sodium arachidonate was purchased from Nu-Chek Prep. Inc. (Elysian, MN). Apyrase, ADP, and human fibrinogen were purchased from Sigma. All other reagents were obtained as previously described [20].

2.2. Platelet preparation

After informed consent was obtained, venous blood was collected from healthy adult donors who denied having taken any drugs for at least two weeks prior to the donation. Anti-coagulation of blood with acid/citrate/dextrose anticoagulant and preparation of platelet-rich plasma (PRP) were performed as described previously [21]. PRP was incubated with 1 mM ASA for 30 min at 37°C. Gel-filtered platelets were prepared as described by Tangen et al. [22] and finally suspended at a concentration of 1×10^9 cells/ml in HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, 1 mg/ml BSA, 3.3 mM NaH_2PO_4, and 20 mM HEPES, pH 7.4).

2.3. Immunoprecipitation, immunoblotting, and in vitro kinase assays

Gel-filtered ASA-treated platelets were incubated with or without various concentrations of TPO for appropriate periods at 37°C. Following the TPO treatment, platelets were lysed for 1 h in a half-volume of 3 × lysis buffer (150 mM NaCl, 15 mM EGTA, 3% Triton X-100, 3% sodium deoxycholate, 0.3% SDS, 3 mM PMSF, 3 mM Na_3VO_4, 60 μg/ml leupeptin, 60 μg/ml aprotinin, and 50 mM Tris-HCl, pH 7.4). This and all subsequent steps were carried out at 4°C. For preparation of whole platelet lysates, the extracts were centrifuged at $10,000 \times g$ for 30 min, diluted with an equal volume of 2 × Laemmli SDS sample buffer, and boiled for 5 min. Immunoprecipitation for JAKs and STATs was done as previously described [20], using 5 μg/ml antibodies specific for each protein and rabbit anti-mouse IgG as a control.

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Immunoprecipitated proteins were eluted with SDS sample buffer and boiled for 5 min. The samples (the whole platelet lysates from 6×10^6 cells/lane or the immunoprecipitates from 2.6×10^8 cells/lane) were subjected to 8% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane (Bio-Rad, Hercules, CA) with a semidry blotter. The membranes were treated for immunoblot assay as described previously [20]. To detect phosphotyrosine-containing proteins, the membranes were incubated with a mAb 4G10 (1 μg/ml). For detection of JAKs and STATs, antibodies (1 μg/ml) against each protein were used as the primary antibodies. In some experiments, the membranes once probed were stripped of bound antibodies and reprobed with other antibodies. In vitro kinase activity of immunoprecipitated JAKs was determined as previously described [23].

2.4. Platelet aggregometry

PRP was prepared from five different volunteers as described above with 0.38% sodium citrate instead of acid/citrate/dextrose anticoagulant. PRP was adjusted at a concentration of 2×10^8 cells/ml and incubated with or without 1 mM ASA for 30 min at 37°C before aggregometry. Gel-filtered platelets suspended at a concentration of 3×10^8 cells/ml in HEPES buffer were also prepared as described above. Standard aggregometory of PRP or gel-filtered platelets with 100 μg/ml human fibrinogen was performed in an aggregometer (HEMA TRACER 1, Nikko Bioscience, Tokyo, Japan).

3. Results

3.1. Induction of protein-tyrosine phosphorylation by recombinant human TPO in human platelets

We first investigated the effect of TPO on tyrosine phosphorylation of platelet proteins in vitro. With anti-phosphotyrosine immunoblot, we found that TPO treatment of gel-filtered platelets induced tyrosine phosphorylation of several proteins in a concentration- and time-dependent manner (Fig. 1A,B). When platelets were exposed to various concentrations of TPO for 3 min, 125-, 98-, and 94/92-kDa tyrosine-phosphorylated protein bands were detected at a minimal concentration of 2 ng/ml and their intensity reached the maximum at 50 ng/ml (Fig. 1A). Interestingly, among the tyrosine-phosphorylated proteins in resting platelets, the phosphotyrosine content of 64-kDa protein band was increased by TPO treatment. Following exposure to 50 ng/ml TPO, tyrosine phosphorylation of these proteins peaked at 2–5 min and slightly diminished at 20 min (Fig. 1B).

3.2. Tyrosine phosphorylation of JAK family kinases, Tyk2 and JAK2, induced by TPO in platelets

We examined tyrosine phosphorylation of four members (Tyk2, JAK1, JAK2, amd JAK3) of the JAK family. Using immunoprecipitation and immunoblotting techniques, we detected all four JAK kinases in platelets (Fig. 2b). The exposure of platelets to TPO yielded tyrosine phosphorylation of Tyk2 (135 kDa) and JAK2 (125 kDa), but not JAK1 (130 kDa) and JAK3 (115 kDa) (Fig. 2a). TPO-induced tyrosine phosphorylation of both Tyk2 and JAK2 were detected at 30 s following TPO (50 ng/ml) treatment, peaked at 2–5 min, and slightly diminished at 20 min (Fig. 3Aa and 3Ba). As it is generally accepted that tyrosine phosphorylation of JAKs is associated with the activation of kinase activity [13,23], TPO treatment increased in vitro kinase activity of Tyk2 and JAK2 in parallel with tyrosine phosphorylation of these kinases (data not shown).

3.3. Tyrosine phosphorylation of STAT3 induced by TPO in platelets

We examined whether TPO induced tyrosine phosphorylation of STATs in platelets. As shown in Fig. 4Ab, we identified STAT1α (91 kDa), STAT2 (113 kDa), and STAT3 (92 kDa). Also, we further detected STAT5 and STAT6, but did neither STAT1β nor STAT4 (data not shown). Among them, incubation of platelets with TPO (50 ng/ml) for 3 min resulted in tyrosine phosphorylation of STAT3 (Fig. 4Aa). Following TPO exposure, phosphorylation of STAT3 on tyrosine was detected at 1 min, peaked at 5 min, and slightly diminished at 20 min (Fig. 4Ba). The kinetics of tyrosine phosphorylation of STAT3 was somewhat slower than those of Tyk2 and JAK2 (Fig. 3A,B).

3.4. TPO-induced ASA-insensitive enhancement of platelet aggregation

Hammond et al. have recently reported that TPO potentiates ADP-induced platelet aggregation and that this activation of platelet function can be blocked by ASA, suggesting that TPO

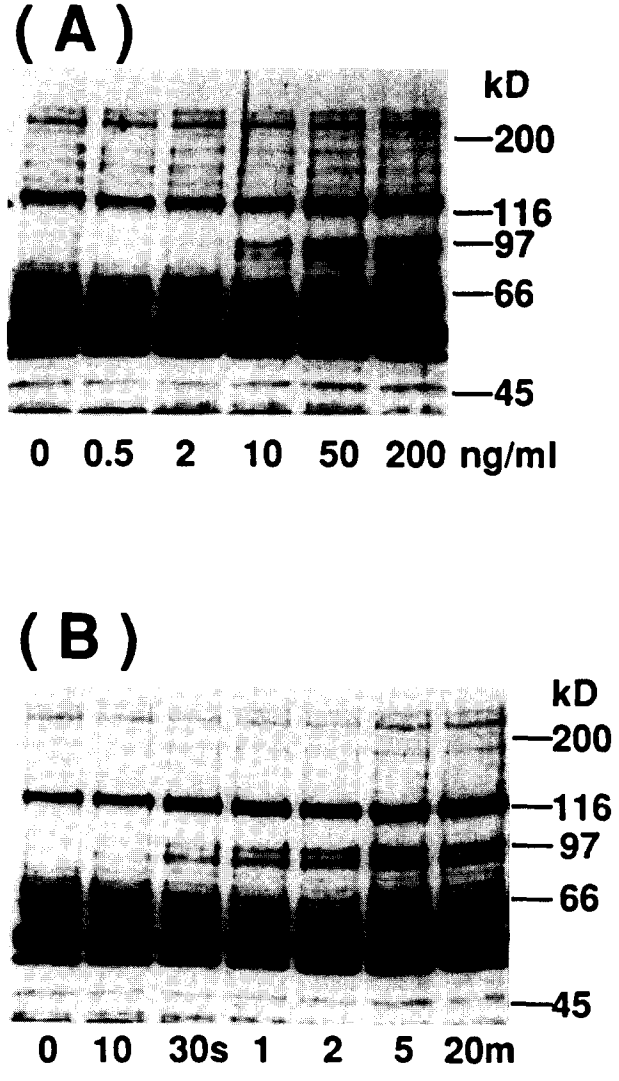


Fig. 1. Dose- and time-dependent tyrosine phosphorylation induced by TPO in platelets. Gel-filtered platelets were incubated with TPO at the indicated concentrations (A) or 50 ng/ml (B), for 3 min (A) or various periods (B). Platelet lysates were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. Molecular mass markers are indicated in kDa.

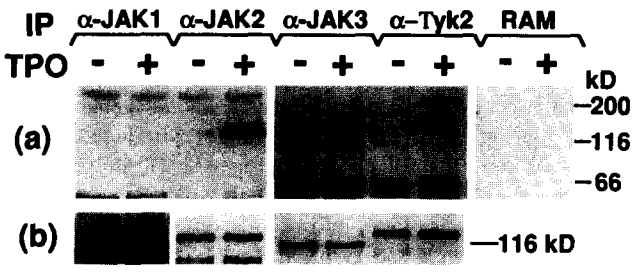


Fig. 2. TPO-induced tyrosine phosphorylation of JAK family kinases. Gel-filtered platelets were incubated with (+) or without (−) 50 ng/ml TPO for 3 min and immunoprecipitated with antibodies against JAK1, JAK2, JAK3, and Tyk2, or rabbit anti-mouse IgG (RAM) as a control. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (a). The immunoblots were then stripped and reprobed with the same antibodies as used for immunoprecipitation (b). Molecular mass markers are indicated in kDa.

potentiates platelet function in an ASA-sensitive way [19]. Therefore, we examined the effects of TPO on platelet function and compared them between ASA-treated and -untreated platelets. TPO by itself did not induce either platelet aggregation or shape change at concentrations up to 1000 ng/ml tested (data not shown). As shown in Fig. 5A, traces a and b, the addition of 5 μM ADP into PRP caused only the primary aggregation which was not affected by ASA treatment. When ASA-untreated platelets were preincubated with 5 ng/ml TPO for 3 min and stimulated by 5 μM ADP, TPO enhanced the stimulatory effect of ADP leading to irreversible aggregation (Fig. 5A, trace e). ASA treatment completely abolished the potentiation effect of 5 ng/ml TPO on 5 μM ADP-induced platelet aggregation (Fig. 5A, trace c), confirming the findings by Hammond et al. However, when platelets were preincubated with 20 ng/ml TPO prior to 5 μM ADP stimulation, ASA treatment failed to change the aggregation curve by the stimulation of TPO plus ADP into that by ADP stimulation alone (Fig. 5A, traces b, d and f). On the other hand, ASA treatment completely abolished 2 mM arachidonate-induced aggregation (data not shown). These suggested to us that TPO at relatively higher concentrations could enhance the primary aggregation, which was characterized by disaggregation and insensitive to ASA treatment. Therefore, we focused on the effects of TPO on the primary aggregation. In order to ensure the primary aggregation alone, platelets were stimulated by low concentrations of ADP or pretreated by ASA (Fig. 5B). Whether platelets were aspirinized or not, pretreatment of platelets with 50 ng/ml TPO resulted in the enhancement of 1 μM ADP-induced primary aggregation (Fig. 5B, traces a and b). Likewise, 50 ng/ml TPO enhanced 5 or 10 μM ADP-induced primary aggregation of aspirinized platelets (Fig. 5B, traces c–f). The potentiation effect on the primary aggregation induced by 5 μM ADP was dependent on TPO concentrations (Fig. 5, traces c and d in panel A, and trace e in panel B). This dose-dependency of TPO effect was confirmed also by various ADP concentrations up to 10 μM, and a concentration of 200 ng/ml TPO elicited the maximal potentiation effect on the primary aggregation (data not shown). The ASA-insensitive enhancement of aggregation by TPO was also observed in the experiments using epinephrine or collagen as agonists (Y. Ezumi, H. Takayama, and M. Okuma, unpublished observations). Furthermore, essentially similar results described above were obtained from the experiments using gel-filtered platelets in the presence of fibrinogen (data not shown).

4. Discussion

Recently, many investigators have reported that protein-tyrosine phosphorylation plays a critical role in the signal transduction through ligand binding to the cytokine receptor superfamily, including c-Mpl [11,14–17,24]. First, we studied tyrosine-phosphorylated proteins in ASA-treated platelets exposed to TPO. Although, we confirmed that TPO induced rapid tyrosine phosphorylation of several proteins including JAK2 in platelets as previously reported by Miyakawa et al. [24], the present work is the first report demonstrating that TPO stimulated tyrosine phosphorylation and kinase activity of Tyk2 in platelets. We also identified for the first time STAT family members, STAT1α, STAT2, STAT3, STAT5, and STAT6, in platelets and demonstrated that TPO stimulated tyrosine phosphorylation of STAT3. Very recent studies have shown that TPO activates STAT1, STAT3, or STAT5 in several cell llines [15–17]. Since platelets are terminal-differentiated anuclear cells, it is tempting to speculate that tyrosine-phosphorylated STAT3 could play a different role in platelets from the roles in other cells reported previously. Other signaling molecules in platelets may interact with STAT3, which has SH2 and SH3 domains and is tyrosine-phosphorylated.

It has been recently reported in an abstract form by Hammond et al. that TPO enhances platelet aggregation induced by ADP and that ASA blocks this activation [19]. We confirmed that ADP-induced platelet aggregation was enhanced by TPO. Their report seems to agree with our findings that ASA inhib-

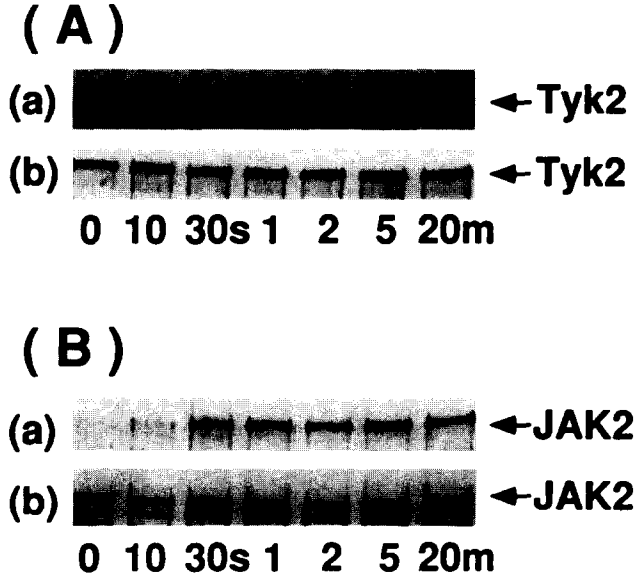
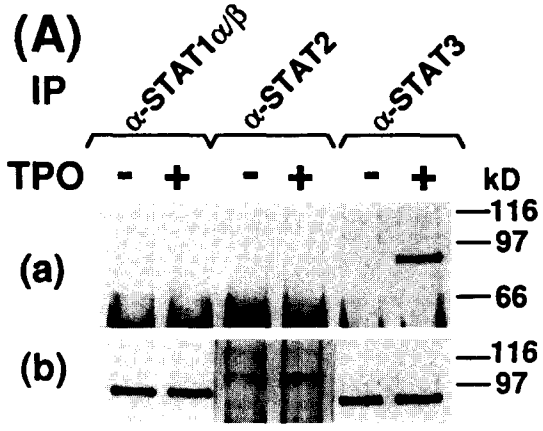


Fig. 3. Time course of TPO-induced tyrosine phosphorylation of Tyk2 and JAK2. Gel-filtered platelets were incubated with 50 ng/ml TPO for the indicated periods and immunoprecipitated with anti-Tyk2 (A) or anti-JAK2 (B) antibodies. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (a in panels A and B). The immunoblots were then stripped and reprobed with anti-Tyk2 (b in panel A) or anti-JAK2 (b in panel B) antibodies. Arrows mark the positions of Tyk2 and JAK2.



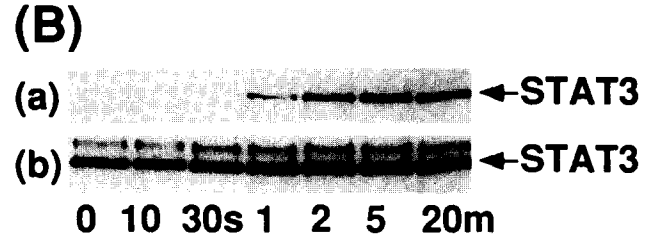


Fig. 4. TPO-induced tyrosine phosphorylation of STAT3. (A) Gel-filtered platelets were incubated with (+) or without (−) 50 ng/ml TPO for 3 min and immunoprecipitated with antibodies against STAT1α/β, STAT2, and STAT3. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (a). The immunoblots were then stripped and reprobed with the same antibodies as used for immunoprecipitation (b). Molecular mass markers are indicated in kDa. (B) Gel-filtered platelets were incubated with 50 ng/ml TPO for the indicated periods and immunoprecipitated with anti-STAT3 antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (a). The immunoblots were then stripped and reprobed with anti-STAT3 antibody (b). Arrows mark the position of STAT3.

ited the irreversible secondary aggregation enhanced by TPO. However, at higher concentrations of TPO than 5 ng/ml, we found that TPO enhanced the primary aggregation, which was characterized by disaggregation and insensitive to ASA treatment, although the secondary aggregation was still inhibited by ASA. This ASA-insensitive potentiation effect of TPO on the primary aggregation was dose-dependent irrespective of ADP concentrations used. Such observations were also obtained with other agonists, collagen or epinephrine, than ADP. Thus, we demonstrated for the first time that TPO potentiated platelet aggregation in an ASA-insensitive way.

There are several pieces of evidence for the modulation of in vitro platelet function by other cytokines or growth factors. IL-1β and IFN-γ enhance the adhesion of thrombin-treated platelets to monocytic leukemia cells, U937, and the release of serotonin by thrombin [25]. G-CSF augments the secondary aggregation of platelets induced by ADP [26]. PDGF inhibited collagen- or thrombin-induced aggregation [27] and secretion of ADP or β-hexosaminidase in an autocrine manner [28]. Stem cell factor, c-Kit ligand, potentiates the secondary wave of aggregation and serotonin secretion induced by ADP and epinephrine [29]. IL-6 enhances agonist-induced aggregation and secretion of thromboxane B_2 via a mechanism involving arachidonic acid metabolism [30]. Like TPO, these cytokines and growth factors described above do not induce aggregation by themselves. The aggregation enhancement by G-CSF, stem cell factor, and IL-6 is observed only in the secondary aggregation. In view of the fact that TPO potentiated not only the secondary aggregation but also the primary aggregation, TPO is unique. The mechanisms for the effects of TPO on platelet function must be different from those of other cytokines and growth factors. TPO might modulate platelet activation possibly through the tyrosine phosphorylation of proteins including JAKs and STATs which TPO can induce regardless of ASA treatment. Considering the clinical application of TPO for the future, it is important to evaluate the influence of in vivo TPO administration on platelet function as well as platelet production.

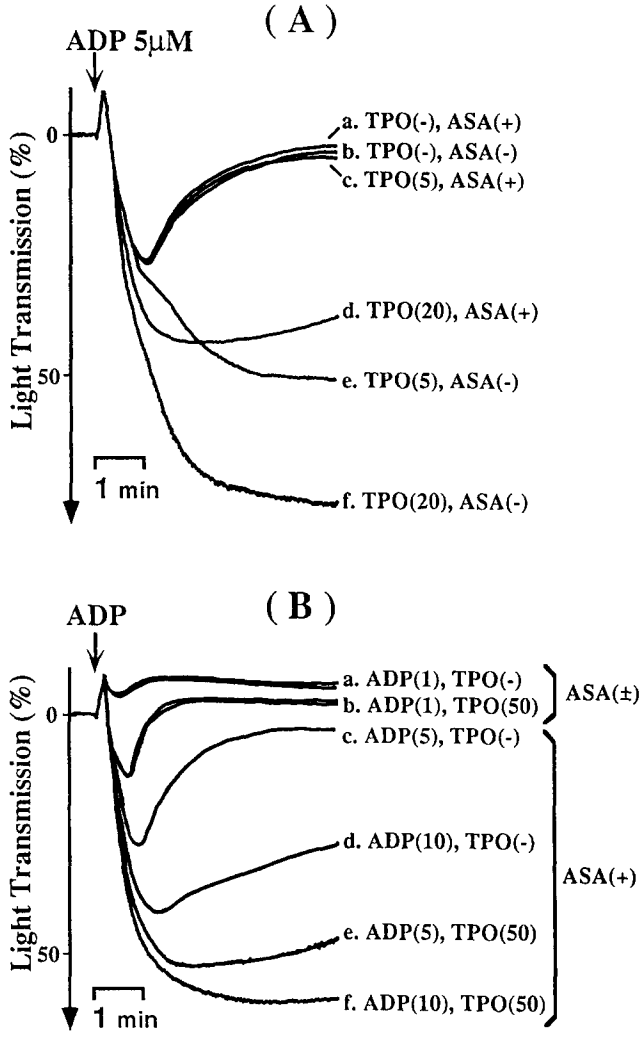


Fig. 5. Effect of TPO on ADP-induced aggregation of PRP pretreated with or without ASA. Citrated PRP was incubated with (+) or without (−) 1 mM ASA for 30 min. Aggregation was monitored using PRP treated with TPO (in ng/ml) or vehicle alone (−) for 3 min prior to the addition of ADP (in μM). Each of symbols a and b in panel B shows two curves overlapped for ASA-treated or -untreated (±) platelets. Results are representative of five independent experiments with essentially similar results.

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