Thrombopoietin potentiates activation of human platelets in association with JAK2 and TYK2 phosphorylation

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Thrombopoietin (TPO), also known as the c-mpl ligand, stimulates rapid tyrosine phosphorylation of multiple proteins in human platelets including the Janus family kinases JAK2 and TYK2. On its own, TPO has no effect on platelet aggregation and dense-granule secretion but induces a general potentiation of these responses by other stimuli. The most dramatic effect is observed against threshold concentrations of agonists for aggregation. Shape change or weak reversible aggregation induced by low concentrations of thrombin, collagen and the thromboxane mimetic, U46619, are converted into irrreversible aggregation in the presence of TPO. A similar result is obtained in the presence of the ADP scavenger apyrase and cyclo-oxygenase inhibitor indomethacin. TPO also induces potentiation of dense-granule secretion measured through release of 5-hydroxy[^{3}H]-tryptamine. This effect is most striking against low concentrations of stimuli and is independent of aggregation as it is observed in the presence of chelation of extracellular Ca^{2+} with EGTA. TPO potentiates activation of phospholipase C and elevation of intracellular Ca^{2+}, providing a molecular explanation for potentiation of functional responses. TPO may have an important physiological role in priming platelet activation in thrombocytopenia, an action that may help to compensate for the reduced platelet density.

INTRODUCTION

Thrombopoietin (TPO), also termed megakaryocyte colony-stimulating factor (Meg-CSF), megakaryocyte growth and development factor (MGDF) and c-mpl ligand, is a novel cytokine that promotes the differentiation and maturation of megakaryocytes *in vitro* [1–3]. Recent reports show that TPO also supports formation and release of platelets from suspension cultures of megakaryocytes [4,5]. *In vivo*, TPO markedly increases circulating platelet levels in association with an increase in megakaryocytes and their progenitors [1,2,6,7]. A direct relationship between the level of TPO and platelet mass in thrombocytopenic rabbits has been described [8]. Kaushansky proposed a model of regulation of thrombocytopoiesis where TPO plays a crucial role in the development and maturation of megakaryocytes, acknowledging a contribution from other cytokines [9].

TPO is the ligand for the c-mpl receptor, the cellular homologue of the v-mpl oncogene that causes an acute myeloproliferative syndrome in mice [10–13]. The c-mpl receptor is localized to early haematopoietic cells, megakaryocytes and platelets [14,15]. It shares high structural and amino acid similarity with the erythropoietin (EPO) receptor [11]. Many cytokines induce rapid tyrosine phosphorylation of intracellular proteins through activation of the Janus kinase (JAK) family of tyrosine kinases [16–18]. Four members of the family have been identified, JAK1, JAK2, JAK3 and TYK2. There is evidence for a role of JAK2 in signalling by TPO in M07e megakaryocytic cells [19] and Ba/F3 cells expressing the murine c-mpl receptor [20]. Studies using mutant TPO receptors in Ba/F3 cells have shown that the ability of TPO to induce mitogenesis correlates with activation of JAK2 [21].

TPO represents a new therapeutic agent in the treatment of thrombocytopenias offering an alternative to platelet transfusion or the administration of other more pleiotropic cytokines such as interleukins 3 and 11. It is therefore important to characterize the functional effect of TPO on platelets. Recently, two abstracts have reported potentiation of platelet aggregation induced by ADP and dense-granule secretion induced by a variety of agonists in the presence of TPO [22,23]. In the present study, we have characterized in more detail the action of TPO on human platelets.

EXPERIMENTAL

Materials

Purified recombinant human TPO, kindly provided by Dr. D. Foster (Zymogenetics Corp., Seattle, WA, U.S.A.), was produced in baby hamster kidney (BHK) cells grown in serum-free medium [6]. The supernatant was used as a source of TPO and its activity was quantified in a c-mpl ligand-dependent cell line. The dilution that caused half-maximal proliferation was defined as 10 units/ml (Dr. Foster, personal communication). Affinity-purified anti-JAK2 and anti-TYK2 were obtained from Santa Cruz Biotechnology (NBS Biologicals, Hatfield, Herts., U.K.). The anti-phosphotyrosine monoclonal antibody (mAb), 4G10, and the antiserum against JAK2 were from Upstate Biotechnology Inc. (TCS Biologicals Ltd., Bucks., U.K.). Antiserum against JAK3 was a gift from Dr. John O'Shea (National Cancer Institute, Frederick, MD, U.S.A.). mAb IV.3 was purchased from Madarex Inc. (West Lebanon, NH, U.S.A.). Nonidet P40 (NP40) was purchased from BDH (Poole, Dorset, U.K.). Collagen was from Hormon-Chemie (Munich, Germany). Thrombin, phorbol 12,13-dibutyrate (PDBu), U46619 (11α,9α-epoxymethanoprosta-

glandin H$_2$) and apyrase were from Sigma (Poole, Dorset, U.K.). Ro 31-8220 was a gift from Roche Products (Welwyn Garden City, Herts., U.K.). myo-[^{3}H]Inositol (18.2 Ci/mmol) and 5-hydroxy[^{3}H]tryptamine (5-HT; 25 Ci/mmol) were from Amersham International (Cardiff, U.K.). Fura-2-AM was from Molecular Probes (Eugene, OR, U.S.A.). Other reagents were obtained from sources described previously [24].

Platelet isolation and measurement of secretion and aggregation responses

Human platelets were isolated from aspirin-free volunteers on the day of the experiment and resuspended at a concentration of $(0.4–1.6) \times 10^9$ cells/ml in a modified Tyrodes-Hepes buffer as described previously [25]. EGTA (1 mM), indomethacin (10 μM) or apyrase (0.2 unit/ml) were included as required. In studies involving measurement of 5-HT release, platelets were prelabelled with [^{3}H]5-HT in plasma [25]. Platelet suspensions (0.4 ml) were prewarmed at 37 °C for 5 min in a Born aggregometer with stirring at 800 rev./min before addition of test compounds. The low-affinity IgG receptor, Fcγ receptor IIA (FcγRIIA), was stimulated by cross-linking with mAb IV.3 (1 μg/ml) added 60 s before F(ab')$_2$ anti-(mouse IgG) (30 μg/ml). Where appropriate, the whole sample was removed at the end of the experiment for measurement of 5-HT secretion as described [26]. The effect of TPO was compared with medium from control BHK cells.

Immunoblotting

An equal volume of Laemmli sample buffer was added to the platelet sample and the mixture heated for 5 min at 100 °C. Proteins were separated by 10 % SDS/PAGE and transferred to poly(vinylidene difluoride) (PVDF) blotting membranes by semi-dry transfer (120 min, 15 V). Non-specific binding was blocked with Tris-buffered saline–Tween (TBS-T: 20 mM Tris/HCl, 137 mM NaCl, 0.1 % Tween 20, pH 7.6) containing 10 % BSA (w/v). Membranes were incubated with the primary antibody dissolved in the same buffer (1 μg/ml anti-phosphotyrosine 4G10, 1:1000 dilution anti-JAK2 and anti-JAK3 sera and 0.1 μg/ml anti-TYK2 antibody) for 60 min at room temperature. Membranes were washed five times in TBS-T before detection of bound antibody with horseradish-peroxidase-conjugated sheep anti-(mouse IgG) (NA 931) or donkey anti-(rabbit IgG) (NA 934) depending on the nature of the first antibody. After washing, blots were treated with enhanced chemiluminescence (ECL) reagents and exposed to Hyperfilm. Membranes were stripped of bound antibody by washing in TBS-T containing 2 % SDS for 40 min at 80 °C. After verifying stripping by use of the secondary antibody, blots were reprobed with a different primary antibody as appropriate.

Immunoprecipitation

Proteins were immunoprecipitated from 500 μl of platelets $(1.6 \times 10^9$/ml) under non-denaturing conditions using an equal volume of either 2 % Triton X-100 or 1 % NP40 in extraction buffer (300 mM NaCl, 20 mM Tris, 1 mM PMSF, 10 mM EDTA, 2 mM Na$_3$VO$_4$, 20 mg/ml leupeptin, 20 μg/ml aprotinin and 5 μg/ml pepstatin). Immunoprecipitations were carried out with 0.5 μg/ml anti-JAK2 and anti-TYK2 antibodies, and 5 μl of anti-JAK3 serum per sample.

Measurements of intracellular Ca^{2+} levels

Platelets were loaded with 3 μM fura-2/AM in platelet-rich plasma for 1 h at 30 °C and resuspended at 4×10^8 cells/ml in a modified Tyrode-Hepes buffer in the presence of 10 μM indomethacin. Fluorescence intensities were measured at excitation wavelengths of 340 nm and 380 nm with emission at 510 nm using a Perkin-Elmer LS50B spectrofluorimeter. Calculation of intracellular Ca^{2+} concentration from the fluorescence ratio (340:380 nm) was performed by use of a previously established calibration curve using Ca^{2+} standards [27].

Measurement of [^{3}H]inositol phosphates

Platelets were labelled with 50 μCi/ml of [^{3}H]inositol for 3 h at 30 °C and resuspended at a concentration of 4×10^8 cells/ml in the presence of 10 μM indomethacin and 0.2 unit/ml apyrase. LiCl (10 mM) was added to each sample to inhibit conversion of inositol phosphates into free inositol. Reactions were stopped after 3 min of platelet stimulation with 0.94 ml of chloroform/methanol/HCl (50:100:1, by vol.); water (0.31 ml) and chloroform (0.31 ml) were added. After separation of phases, total [^{3}H]inositol phosphates (mono-, bis- and trisphosphates) were eluted by Dowex anion-exchange chromatography as described [28].

Analysis of results

Statistical analyses were performed by Student's t-test, with $P < 0.05$ taken as the level of significance.

RESULTS AND DISCUSSION

TPO induces tyrosine phosphorylation of JAK2 and TYK2 in human platelets

TPO (400 units/ml) stimulates rapid tyrosine phosphorylation of multiple platelet proteins with the most prominent increase observed at 125 kDa with a doublet at 90 kDa (Figure 1). The increase in phosphorylation was rapid, peaking at between 5 and 15 min, and was maintained for at least 30 min (results not shown). A weakly tyrosine-phosphorylated 38 kDa band was detected at 15 min but not at earlier times (Figure 1). The control BHK medium did not increase tyrosine phosphorylation or
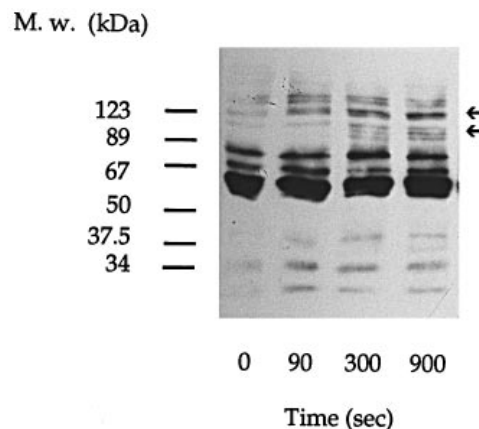

Figure 1   TPO induces tyrosine phosphorylation of several proteins in human platelets

Samples of platelets (4×10^8/ml) were stimulated with TPO (400 units/ml) for the times indicated. Samples were heated for 5 min at 100 °C in Laemmli buffer. Proteins were separated by 10% SDS/PAGE, transferred to PVDF membranes by semi-dry transfer and immunoblotted with the anti-phosphotyrosine mAb 4G10. Similar results were seen in three other experiments.
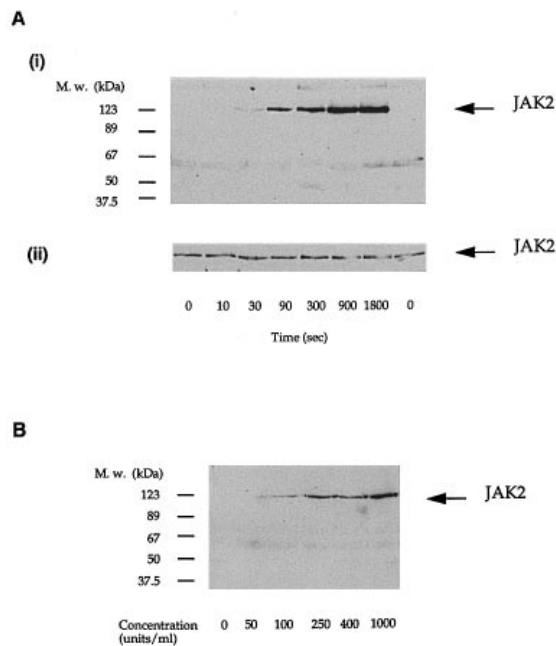
Figure 2   Tyrosine phosphorylation of JAK2 induced by TPO

(**A**) (i) Time-course of tyrosine phosphorylation of JAK2 stimulated by TPO (250 units/ml). Platelets (1.6×10^9/ml) were challenged with TPO at 37 °C for the time indicated. The lane on the left (labelled zero) corresponds to a sample treated with buffer while the lane on the right (also labelled zero) corresponds to a sample treated with the control BHK cells medium. Cells were lysed in non-denaturing conditions (1 % Triton X-100) and protein immunoprecipitated with 0.5 μg/ml anti-JAK2 antibody from Santa Cruz Biotechnology. The immunoprecipitates were submitted to SDS/PAGE and protein was transferred to membrane and probed with anti-phosphotyrosine antibody 4G10. (ii) The same membrane was stripped and reprobed with the antiserum against JAK2 from Upstate Biotechnology Inc. (UBI). Results are representative of two other experiments. (**B**) Concentration-dependent tyrosine phosphorylation of JAK2 induced by TPO. Platelets were stimulated with TPO for 5 min at the concentrations indicated. JAK2 was immunoprecipitated as detailed in (**A**). Results are representative of two other experiments.
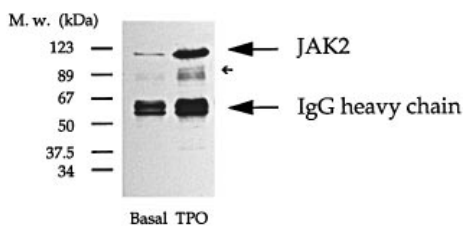

Figure 3   Several tyrosine-phosphorylated proteins co-precipitate with JAK2 after TPO stimulation

Platelets (1.6×10^9/ml) were challenged with 250 units/ml TPO at 37 °C for 5 min. Cells were lysed in non-denaturing conditions (0.5% NP40) and protein immunoprecipitated with 0.5 μg/ml anti-JAK2 antibody from Santa Cruz Biotechnology. The immunoprecipitates were submitted to SDS/PAGE and protein was transferred to membrane and probed with anti-phosphotyrosine antibody 4G10. The small arrow indicates the 90 kDa doublet which co-precipitates with JAK2. The small increase in intensity of the IgG chain was not reproducible between experiments. Results are representative of four other experiments.

mimic the other actions of TPO that are described below (results not shown).

Immunoprecipitation studies using a specific anti-JAK2 antibody revealed that TPO induces tyrosine phosphorylation of JAK2 (Figures 2Ai and 3). TPO-induced phosphorylation could be detected at 30 s, peaked at 15 min and was maintained for
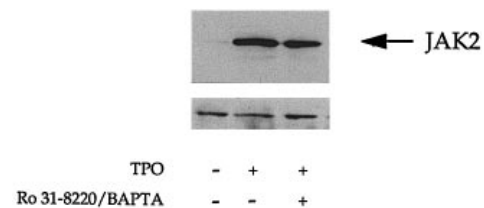

Figure 4   Effect of PKC inhibition and intracellular calcium chelation on tyrosine phosphorylation of JAK2 stimulated by TPO

Platelets were treated with 10 μM Ro 31-8220 for 1 min and 40 μM BAPTA-AM for 15 min, and then exposed to 250 units/ml TPO for 5 min. JAK2 was immunoprecipitated from the lysed cells with 0.5% NP-40 and immunoblotted with the anti-phosphotyrosine antibody 4G10 (upper panel). 4G10 was removed by stripping as detailed in the Experimental section and reprobed with the anti-JAK2 serum (lower panel). Results are representative of two experiments run in duplicate.

30 min (Figure 2A). Reprobing the membrane with a different anti-JAK2 serum demonstrated that similar levels of protein were immunoprecipitated at all time points (Figure 2A). Tyrosine phosphorylation of JAK2 by TPO was concentration-dependent (Figure 2B), with a threshold of 50 units/ml and peaking at 1000 units/ml. This corresponds to the concentration–response curve of phosphorylation of the 125 kDa protein in whole-cell lysates (results not shown). Consistent with this, immunoprecipitation of JAK2 in the presence of NP40 depleted the 125 kDa protein observed in whole-cell lysates by more than 90 % (results not shown). Rapid tyrosine phosphorylation of JAK2 by TPO in platelets has also been reported [29] and has been observed in Ba/F3 cells transfected with the c-mpl receptor [20].

Several tyrosine-phosphorylated proteins, including a prominent doublet at 90 kDa, co-precipitate with JAK2 in platelets stimulated by TPO following extraction in the presence of NP40 (Figure 3). A phosphorylated protein of 38 kDa is also seen in longer exposures of the blot. The 90 kDa doublet may contain members of the STAT (signal transducers and activators of transcription) family of transcription factors as these are known to be substrates for JAK kinases in a number of cytokine receptor systems [17,18]. The presence of additional proteins in NP40 but not Triton X-100 extracts may be due to a greater level of immunoprecipitation of JAK2 in the presence of NP40. Triton X-100 is a relatively mild detergent which is unable to extract proteins from the cytoskeleton. Alternatively, association of these proteins with JAK2 may not be stable in the presence of Triton X-100.

We have reported previously that JAK2 is phosphorylated on tyrosine in thrombin-stimulated platelets through a pathway that is downstream of phosphoinositide metabolism since it is inhibited by Ro 31-8220, a protein kinase C inhibitor, and 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid acetoxymethyl ester (BAPTA-AM), an intracellular Ca^{2+} chelator [24]. The combination of Ro 31-8220 and BAPTA-AM causes a small reduction in tyrosine phosphorylation of JAK2 induced by TPO (5.1 ± 0.9 %, $n = 4$) (Figure 4). The small degree of inhibition may reflect a toxic action of BAPTA-AM as reported by others [30], in view of the fact that TPO does not stimulate phospholipase C (PLC) or raise intracellular Ca^{2+} in platelets (see below). JAK2 phosphorylation by TPO may involve direct association with the c-mpl receptor by analogy with the mechanism underlying phosphorylation induced by EPO [17].

The effect of TPO on tyrosine phosphorylation of other members of the Janus family of kinases was investigated through immunoprecipitation studies using specific antibodies. TPO in-
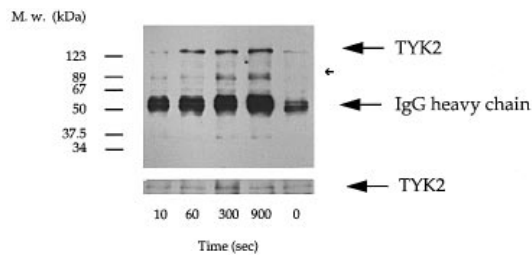
Figure 5   Tyrosine phosphorylation of TYK2 induced by TPO

Platelets (1.6×10^9/ml) were challenged with 250 units/ml TPO at 37 °C for the time indicated and lysed in non-denaturing conditions using 0.5 % NP-40. Protein was immunoprecipitated with 0.5 μg/ml anti-TYK2 antibody from Santa Cruz Biotechnology. The immunoprecipitates were divided in half and submitted to SDS/PAGE. After the transfer, one membrane was probed with anti-phosphotyrosine antibody 4G10 (upper panel) and the other with the antibody against TYK2 from Upstate Biotechnology Inc. (UBI) (lower panel). The small arrow indicates the 90 kDa doublet that co-precipitates with TYK2. Results are representative of two other experiments.

duces rapid tyrosine phosphorylation of TYK2 over a similar time course to that for phosphorylation of JAK2 (Figure 5). Immunoprecipitation using NP40 also revealed co-precipitation of a tyrosine-phosphorylated doublet at 90 kDa, which may be the same as that observed in JAK2 immunoprecipitates, together with additional proteins that co-migrate with the IgG heavy chain. TPO did not induce tyrosine phosphorylation of JAK3 (results not shown). We were unable to detect the presence of JAK1 in platelets using commercial antibodies [24]. Although participation of two members of the JAK family of tyrosine kinases in signalling by cytokines has been described, receptors with a strong structural resemblence to the TPO receptor such as those for EPO, granulocyte colony-stimulating factor (G-CSF), growth hormone and prolactin are believed to signal through JAK2 [31–33]. Further work is required to define the contribution of TYK2 and JAK2 to receptor signalling elicited by TPO.

TPO primes platelet functional responses

On its own, TPO (10–1000 units/ml) has no effect on platelet shape change, aggregation or secretion following incubation for periods up to 30 min. On the other hand, TPO induces potentiation of secretion and aggregation responses to a wide range of agonists. The effect on aggregation is particularly dramatic. Shape change or weak, reversible aggregation responses induced by collagen, thrombin and the thromboxane mimetic U46619 are converted into irreversible aggregation in the presence of TPO (Figures 6A–6D). TPO has little effect on aggregation induced by higher concentrations of each stimulus, possibly because of the rapid nature of the response. The concentration–response relationship for potentiation of aggregation is similar to that for phosphorylation of JAK2 with a threshold at ~100 units/ml. Potentiation is rapid in onset and maximal between 5 and 15 min. The pattern of aggregation induced by the phorbol ester, PDBu, is distinct from that induced by the above stimuli in that it is not preceded by a shape-change response. TPO induces potentiation of aggregation by PDBu (Figure 6) and has also been reported to potentiate aggregation induced by ADP [22].

In order to investigate the role of thromboxane formation and ADP release in the potentiating action of TPO on aggregation, experiments were performed in the presence of the ADP scavenger apyrase and cyclo-oxygenase inhibitor indomethacin. The concentration–response curve to thrombin for aggregation was shifted slightly to the right in the presence of the inhibitors,
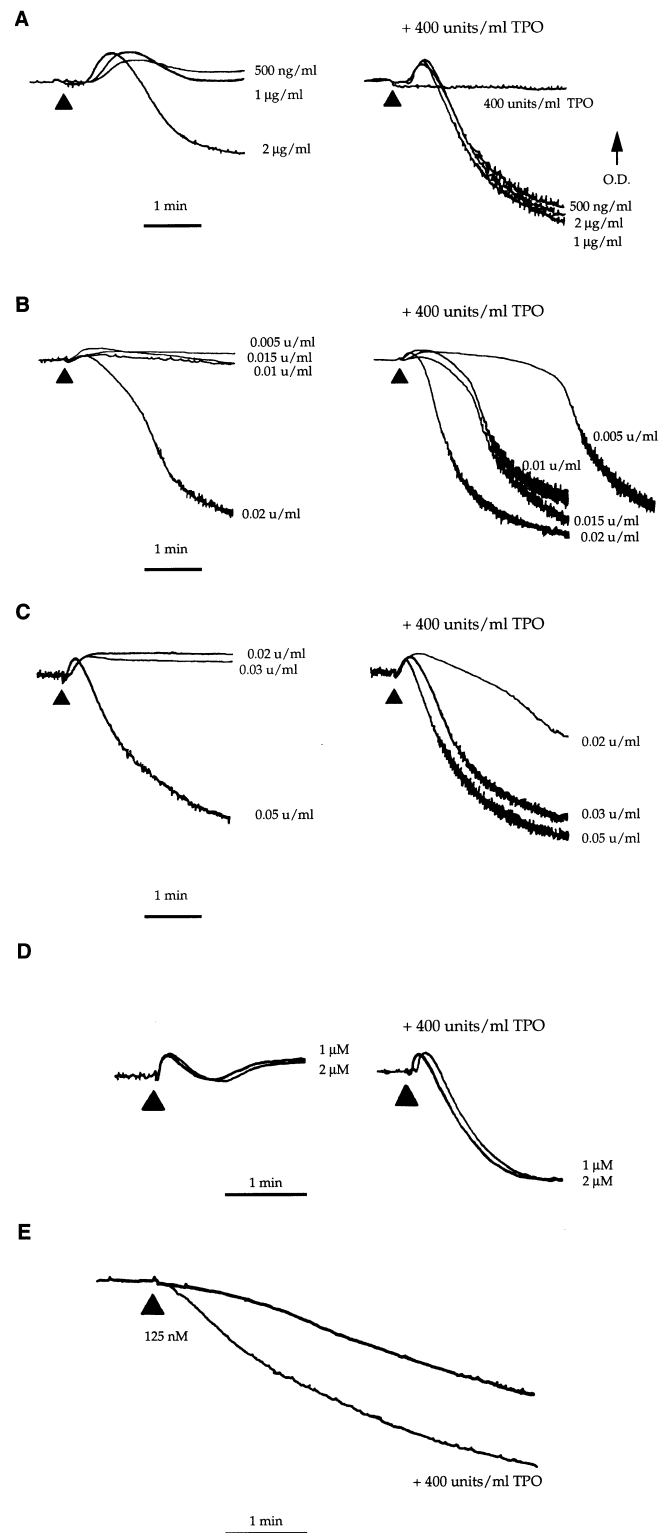

Figure 6   Effect of TPO on aggregation

Platelets were isolated as described in the Experimental section and resuspended at 4×10^8/ml in a modified Tyrodes buffer in the absence (**A,B**) or presence (**C–E**) of 10 μM indomethacin and 0.2 unit/ml apyrase. Where indicated platelets were incubated for 5 min in the presence of 400 units/ml TPO and then stimulated with (**A**) collagen, (**B,C**) thrombin, (**D**) U46619 and (**E**) PDBu at the concentrations indicated. Aggregation measurements were carried out in a Born lumi-aggregometer and are represented as an increase in absorbance. This trace is representative of three experiments.

Table 1   TPO potentiates release of [³H]5-HT

[³H]5-HT-labelled platelets (4×10^8/ml) resuspended in the presence of 10 μM indomethacin were challenged with collagen (10 μg/ml) for 90 s, thrombin (0.02 unit/ml) for 60 s or FcγRIIA cross-linking for 90 s. FcγRIIA was stimulated by cross-linking with mAb IV.3 (1 μg/ml) added 60 s before F(ab′)$_2$ anti-(mouse IgG) (30 μg/ml). Results are shown as means $\pm$ S.E.M. of three experiments run in duplicate and represent percentage release of total cellular [³H]5-HT. Statistical significance: $*P < 0.05$; $**P < 0.01$.

	Release of total cellular [³H]5-HT (%)	
	Control	TPO (400 units/ml)
Basal	–	-0.5 ± 1.3
Collagen (10 μg/ml)	3.9 ± 1.7	$10.1 \pm 1.1**$
FcγRIIA cross-linking	9.4 ± 2.4	$14.2 \pm 2.9*$
Thrombin (0.02 unit/ml)	3.3 ± 0.2	$12.2 \pm 0.2**$

Figure 7   TPO potentiates agonist-induced Ca²⁺ elevation

Platelets were loaded with fura-2 as described in the Experimental section. Platelets were incubated for 5 min in the absence (left-hand traces) or presence (right-hand traces) of 1000 units/ml TPO and then stimulated with (**A**) 30 μg/ml collagen, (**B**) FcγRIIA cross-linker and (**C**) 0.05 unit/ml thrombin. The elevation of intracellular Ca²⁺ is measured as the fluorescence ratio (340/380 nm) and calibrated using Ca²⁺ standards. Traces are representative of two other experiments that gave similar results. The arrowheads indicate the addition of agonist.

consistent with an important role for ADP and thromboxanes in mediating aggregation induced by low concentrations of the protease (Figures 6B and 6C). The potentiating effect of TPO on aggregation was still apparent, however, under these conditions, demonstrating that it is mediated through a direct effect on thrombin-induced signalling events.

TPO causes a small but significant potentiation of [³H]5-HT secretion induced by collagen, thrombin and the platelet low-affinity receptor for immune complexes, FcγRIIA, in the presence of indomethacin (Table 1). Potentiation is observed throughout the whole length of the concentration–response curve to thrombin, but is most apparent against threshold concentrations (results not shown). Potentiation is also observed under conditions in which aggregation is inhibited by inclusion of EGTA (results not shown).
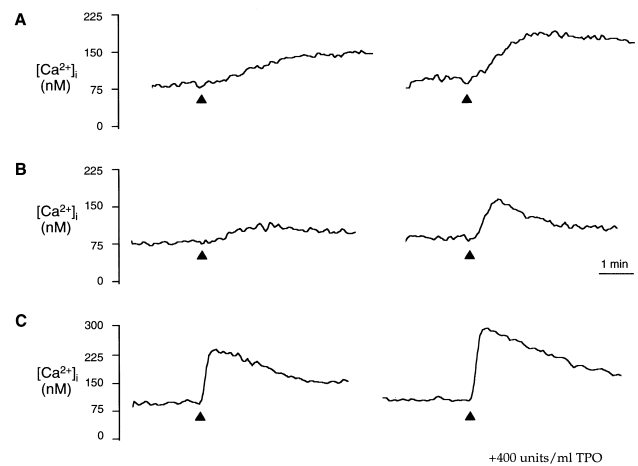
G-CFS and c-Kit ligand (also known as human stem cell factor) have also been reported to potentiate aggregation induced by ADP and adrenaline/ADP respectively, while having no effect on their own [34,35]. c-Kit ligand was also reported to potentiate 5-HT release in adrenaline/ADP-stimulated platelets. The effect of c-Kit ligand is distinct from that of TPO in that it is dependent on the formation of thromboxanes [35]. The mechanism underlying potentiation induced by G-CFS may be similar to that of TPO as their receptors share many features including activation of JAK2 [31,36].

Secretion and aggregation responses to G-protein and tyrosine kinase-linked receptors such as thrombin and collagen are regulated downstream of PLC. On its own, TPO had no effect on PLC activity as determined by measurement of inositol phosphates. On the other hand, a concentration of TPO that converted

the response to a low concentration of thrombin (0.02 unit/ml) from shape change into irreversible aggregation induced a significant increase in PLC activity (Table 2). This effect is independent of ADP secretion or thromboxane formation as it is observed in the presence of apyrase and indomethacin. TPO also potentiates activation of PLC by collagen (results not shown). This increase in PLC activity by thrombin and collagen occurs in parallel with a small elevation in intracellular Ca²⁺ measured in platelets loaded with the fluorescent dye fura2 (Figure 7); a similar result is seen in platelets stimulated by FcγRIIA cross-linking (Figure 7). On its own TPO did not modify the level of intracellular Ca²⁺ (results not shown). The increases in PLC and Ca²⁺ provide a molecular basis for the ability of TPO to potentiate aggregation and secretion. The ability of TPO to also potentiate aggregation to PDBu, which does not induce a detectable elevation of intracellular Ca²⁺ in platelets, however, suggests that additional mechanisms may also play a role in the action of TPO. The similar concentration–response relationships for potentiation of aggregation, secretion and PLC activation by TPO with those for phosphorylation of JAK2 and TYK2 provides evidence for a causal link. Additional work is required to substantiate this possibility.

Conclusions

TPO causes a general potentiation of aggregation and secretion *in vitro* in association with JAK2 and TYK2 phosphorylation. This effect appears to have greatest significance against threshold concentrations of stimuli and may be of physiological importance in thrombocytopenic states. The plasma level of TPO is inversely related to platelet number and this potentiatory action may compensate for the reduced platelet density. It is essential to perform studies *in vivo* to determine the significance of this effect in view of the potential clinical use of TPO in the treatment of thrombocytopenia.

Table 2   TPO potentiates thrombin-induced formation of inositol phosphates

[³H]Inositol-labelled platelets (4×10^8/ml) resuspended in the presence of 10 μM indomethacin and 0.2 unit/ml apyrase were challenged with different concentrations of thrombin for 3 min. Results are measured in d.p.m. and shown as means $\pm$ S.E.M. from one representative experiment performed in quadruplicate which is representative of two similar experiments. Statistical significance: $*P < 0.05$; $**P < 0.01$.

Concentration (units/ml)	Control	TPO (400 units/ml)
0	147.0 ± 11.8	142.1 ± 21.1
0.01	239.7 ± 5.8	235.7 ± 13.7
0.02	276.0 ± 18.2	$512.7 \pm 11.5**$
0.03	612.7 ± 11.9	$704.0 \pm 47*$

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