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Calcium as a mediator between erythropoietin and protein tyrosine phosphatase 1B

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ABSTRACT

Erythropoietin (Epo) is crucial for promoting the survival, proliferation, and differentiation of mammalian erythroid progenitors. The central role played by tyrosine phosphorylation of erythropoietin receptor (EpoR) in Epo-cell activation has focused attention on protein tyrosine phosphatases (PTPs) as candidates implicated in the pathogenesis of the resistance to therapy with human recombinant Epo. Prototypic member of the PTP family is PTP1B, which has been implicated in the regulation of EpoR signaling pathways. In previous reports we have shown that PTP1B is reciprocally modulated by Epo in undifferentiated UT-7 cell line. However, no information is available with respect to the modulation of this phosphatase in non-Epo depending cells or at late stages of erythroid differentiation. In order to investigate these issues we induced UT-7 cells to differentiate and studied their PTP1B expression pattern. Simultaneous observations were performed in TF-1 cells which can be cultured either with GM-CSF, IL-3 or Epo. We found that Epo induced PTP1B cleaveage in TF-1 and differentiated UT-7 cells. This pattern of PTP1B modulation may be due to an increased TRPC3/TRPC6 expression ratio which could explain the larger and sustained calcium response to Epo and calpain activation in Epo treated TF-1 and differentiated UT-7 cells.

Introduction

Erythropoietin (Epo)[1] is a hematopoietic cytokine that regulates the growth and differentiation of erythroid progenitor cells through the activation of its specific receptor (EpoR) [1]. Upon Epo binding, EpoR undergoes phosphorylation on tyrosine residues in the cytoplasmic domain and thereby recruits different SH2-containing signaling molecules, such as STAT5, Shc, SHP-2, and the p85 regulatory subunit of PI3K to activate various signal transduction pathways, most of which are shared with other members of the cytokine receptor family [2,3]. The downregulation of the EpoR signal is attributed to many proteins such as SOCS, inositol phosphatases, and tyrosine phosphatases [4].

PTP1B is a ubiquitously expressed non-receptor protein tyrosine phosphatase, located in the cytosol and on intracellular membranes via its hydrophobic C-terminal targeting sequence [5]. In addition to its role in the downregulation of the EpoR signaling pathway, it was suggested that PTP1B action is essential in the regulation of many cellular functions including cell adhesion [6,7], cellular stress response, and cytokine receptor signaling [8,9]. Since PTP1B is involved in several important cellular pathways, it is thoroughly regulated at different levels. One of the mechanisms of PTP1B post-translational regulation is calpain-dependent enzyme cleavage [10]. The term "calpain activity" generally refers to the combined effects of two cysteine proteases, which are calcium-dependent intracellular enzymes active at neutral pH. Evidence suggests that calpains are involved in a wide variety of cellular processes, including cell motility, cell proliferation, apoptosis, cytoskeleton rearrangement, and hemostasis [11]. The precise mechanism that regulates calpain activation by calcium remains poorly understood mainly due to uncertain local fluctuations in the calcium gradient from agonist-induced entry of extracellular calcium as well as calcium release from intracellular stores. Kuchay et al. demonstrated that PTP1B is a physiological target of calpain-1 in platelets, and suggested that a similar mechanism may regulate calpain-mediated tyrosine dephosphorylation in other cells.

Epo is one of the well known modulators of intracellular calcium concentration ($[Ca]_i$). It induces a dose-dependent increase in $[Ca]_i$ in normal human BFU-E-derived erythroblasts through a voltage-independent ion channel permeable to calcium [12,13] that depends on tyrosine phosphorylation [14]. Recent studies demonstrated that Epo modulates TRPC3, a member of the transient receptor potential channel (TRPC) superfamily, through

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[1] *Abbreviations used:* $[Ca]_i$, intracellular calcium concentration; Epo, erythropoietin; EpoR, erythropoeitin receptor; GM, granulocyte–macrophage colony-stimulating factor; H, hemin treated cells; PTP1B, protein tyrosine phosphatase 1B; TRPC, transient receptor potential channel; U, undifferentiated UT-7 cells.

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a mechanism requiring the activation of phospholipase C (PLCγ) and the interaction of TRPC3 with PLCγ and inositol 1,4,5-triphosphate receptor (IP_3R) [15]. TRPC3 activation by Epo was found to be regulated by TRPC6, another member of this family, acting through signaling mechanisms involving reduced interaction of TRPC6 with PLCγ and EpoR [16]. Both TRPC3 and TRPC6 channels, involved in Epo-modulated calcium influx, are expressed in human erythroid precursors as well as in erythroleukemia cell lines. The TRP superfamily is a diverse group of calcium-permeable cation channels expressed on non-excitable mammalian cells related to the archetypal *Drosophila* Trp and has been divided into six subfamilies [17–19]. TRP channels participate in many physiological functions in eukaryotes and are known to be involved in a number of diseases [18].

In a previous report, we have demonstrated that Epo induces PTP1B expression and increases its catalytic activity in undifferentiated UT-7 cells [20]. Since PTP1B has been related to cell differentiation [21–23], we were interested in the possible influences of erythroid differentiation on Epo-modulated PTP1B expression.

Therefore, this study compared the regulatory mechanisms exerted by Epo on PTP1B in differentiated and undifferentiated UT-7 cells. It also used TF-1 cells to analyze whether the relationship between Epo and PTP1B may be affected by the dependence on different growth factors to achieve cell proliferation, particularly focusing on the role of calpain.

Materials and methods

Materials

All chemicals used were of analytical grade. Bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium o-vanadate, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin A, polyclonal anti-actin (A4700), and EGTA were obtained from Sigma–Aldrich; Iscove's Modified Dulbecco's Medium (IMDM), Fluor-4/acetomethylester (Fluo-4, AM), $MgCl_2$, Taq DNA polymerase, dNTPs, and specific primers for PTP1B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Invitrogen Life Technologies; polyclonal anti-PTP1B antibody (SC-14021, raised against amino acids 301–435 mapping at the C-terminus of PTP1B) from Santa Cruz Biotechnology; calcium ionophore A23187, calpeptin (calpain inhibitor), monoclonal anti-PTP1B antibodies (PH-01, epitope: PTP1B active site, N-terminus and PH-02 epitope: PTP1B C-terminus) from Calbiochem; polyclonal anti-phosphotyrosine (anti-PY) antibody (P-11120) and Protein A-agarose from BD Transduction Laboratories; and Anti-TRPC3 and anti-TRPC6 antibodies from Alomone Labs; nitrocellulose (NC) membranes (Hybond), chemiluminescent system kit (ECL), and Ready To Go T-Primed First-Strand Kit from Amersham Bioscience; agarose from Promega; ethidium bromide from Mallinckrodt; Sybr Green I nucleic acid stain from Roche; fetal bovine serum (FBS) (Bioser) and penicillin–streptomycin (PAA Laboratories) from GENSA; recombinant human erythropoietin (rhuEpo, Hemax) from Biosidus.

Cell line and culture

UT-7 cell line was kindly provided by Dr. Patrick Mayeux (Cochin Hospital, Paris, France). Initially established from bone marrow cells obtained from a patient with acute megakarioblastic leukemia, this cell line shows complete growth dependence on Epo.

Cells were maintained in IMDM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 U/ml Epo, by replacement of medium every 3–4 days [24]. Erythroid differentiation was induced by 30 μM hemin during 48 h (H).

TF-1 cells were obtained from ATCC (CRL-2003). They were established from a heparinized bone marrow aspiration sample from a patient with severe pancytopenia. The cells are completely dependent on interleukin 3 (IL-3) or granulocyte–macrophage colony-stimulating factor (GM-CSF) for long term growth. They can also be cultured with Epo for short periods (less than 7 days). TF-1 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml anfotericin, and 4 ng/ml GM-CSF, by replacement of medium every 2–3 days [25].

Cell cultures were developed at 37 °C, in an atmosphere containing 5% CO_2 and 100% humidity. Proliferation and cell viability were evaluated by Trypan blue exclusion test and MTT assay.

Protein immunoprecipitation and Western blotting

Cells were washed with ice-cold PBS containing 1 mM sodium o-vanadate and lysed on ice-cold hypotonic lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100), with 1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1.37 μg/ml pepstatin A, in a ratio of 200 μl to 10^7 cells. After 30 min-incubation on ice, cell lysates were clarified by centrifugation at 15,000*g* for 15 min at 4 °C. Total proteins were quantified by the Lowry's method [26].

Monoclonal anti-PTP1B antibody (PH-01) was added at 2 μg/ml final concentration and incubated at 4 °C for 1 h with gentle agitation. Protein A-agarose was added and, after overnight incubation with rotation at 4 °C, immunocomplexes were collected by centrifugation at 15,000*g* for 15 min, and washed twice with the lysis buffer.

Immunoprecipitates or cell lysates corresponding to 100 μg of total proteins were boiled for 3 min in the Laemmli sample buffer [27], fractionated on 12% polyacrilamide–SDS gel electrophoresis and electroblotted onto nitrocellulose membrane during 1.5 h (transfer buffer: pH 8.3, 25 mM Tris, 195 mM glycine, 0.05% SDS, and 20% (v/v) methanol). Membranes were blocked by 1 h-incubation in Tris Buffer Saline (25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.1% Tween 20 and 0.5% skim-milk powder, and then incubated with appropriate concentrations of the specific antibody. After washing with TBS – 0.1% Tween 20, the immunoblots were probed with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:1000) for 1 h at 20 °C and washed. Specific antibody signals were detected using the enhanced chemiluminescence system ECL kit and a Fujifilm Intelligent Dark Box II equipment (Fuji) coupled to a LAS-1000 digital camera.

Densitometry with ImageGauge software was performed to quantify TRPC3 and TRPC6 bands. Anti-β-actin polyclonal antibody was used as control for sample loading variations.

Preparation of subcellular fractions

Subcellular fractionation was performed as previously described [28]. Briefly, cells were lysed in hypotonic buffer (5 mM Tris pH 7.4, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA pH 8.0, 1 mM DTT, 10 μg/ml aprotinin, 1 mM PMSF, 10 μg/ml leupeptin, 10 mM benzamidine, and 0.2 mM sodium orthovanadate) at 4 °C for 30 min. After homogenization, samples were centrifuged (2000*g* for 5 min) to remove nuclei, and centrifuged again (10,000*g* for 10 min) to remove the mitochondrial fraction. Supernatants were ultracentrifuged at 50,000*g* for 1.5 h to obtain light membrane fractions which were solubilized in 1% Triton X-100, 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM benzamidine, 1 mM PMSF, and 0.2 mM sodium orthovanadate.

Enzyme activity assay

Aliquots of cell lysate equivalent to 1 mg total protein were immunoprecipitated with polyclonal anti-PTP1B as indicated above and immunoprecipitates washed with PTP assay buffer (18.5 mM HEPES, pH 7.2, 7.5 mM NaCl, 1.85 mM EDTA, 5 mM DTT). The PTP activity was measured in a 200 μl final volume by incubation at 37 °C for 30 min in PTP assay buffer containing 2.4 mM pNPP. Optical density of the product was determined at 415 nm in a microplate reader. The correction for non-enzymatic hydrolysis of pNPP was evaluated by measuring the absorbance in the absence of the enzyme.

Quantitative Real-Time RT-PCR

Real-Time RT-PCR for PTP1B was performed in a volume of 25 μl of a mixture containing cDNA (1:10 dilution), 0.20 mM dNTPs, specific primers at 0.25 μM concentration, 3 mM $MgCl_2$, 2 U Taq DNA polymerase, and 1:30,000 SYBR Green Stain. Real-Time RT-PCR reactions were performed in a DNA Engine Opticon (MJ Research Inc.) and the amplification program consisted of an initial denaturing step (94 °C for 5 min), followed by 40 cycles (each of 94 °C for 45 s, 59.5 °C for 45 s, and 72 °C for 45 s). Sample values were normalized to endogenous GAPDH which was also determined by Real-Time RT-PCR following the same protocol as that for PTP1B. Acquisition of the fluorescence signal from the samples was carried out at the end of the elongation step. Each assay included a DNA minus control and a standard curve performed with serial dilutions of control cDNA obtained from either TF-1 cells maintained with RPMI, 10% FBS, and 2 ng/ml GM, or UT-7 cells maintained with IMDM, 10% FBS, and 2 U/ml Epo. All samples were run in duplicate and the experiment was repeated three times with independently isolated RNA.

Flow cytometry

UT-7 and TF-1 cells were collected by centrifugation and loaded with Fluo-4, AM at 25 °C for 20 min. This colorless calcium indicator enters the cell freely and is hydrolyzed by non-specific intracellular esterases to yield fluorescence when bound to free calcium ions. After washing, cells were incubated in PBS for 15 min and suspended in IMDM (UT-7) or RPMI (TF-1) medium with 1 mM $CaCl_2$. After adding 10 U/ml of Epo, cells were incubated for short periods and analyzed by flow cytometry. Baseline intracellular calcium content was established as the fluorescence obtained from either GM-cultured TF-1 cells or Epo-cultured UT-7 undifferentiated cells. Changes in intracellular calcium content were expressed as percentage of cells with fluorescence above the baseline.

Statistics

Results are expressed as mean ± standard error (SEM). Comparison among groups was carried out by the Kruskal–Wallis one-way analysis of variance and the Mann–Whitney *U*-test when corresponding. Least significant difference with $P < 0.05$ was considered as the criterion for statistical significance.

Results

Epo modulates PTP1B mRNA level and protein cleavage in TF-1 and differentiated UT-7 cells

Since PTP1B expression and catalytic activity are upregulated by Epo [20] and it has been demonstrated that this phosphatase is involved in cell differentiation [21–23], the influence of Epo in the modulation of PTP1B expression was assessed in erythroid differentiated UT-7 cells. UT-7 cell differentiation was induced by hemin and then the possibility that Epo may trigger changes in PTP1B expression was evaluated. To better understand Epo-mediated PTP1B regulation, parallel experiments were carried out with TF-1 cells, a cell line that can be grown in the presence of GM-CSF, IL-3 or Epo. For experimental assays, TF-1 and differentiated UT-7 cells were cultured in the absence of serum and growth factors (Epo or GM-CSF) for 18 h ($t = 0$ h) and, after this starvation period, they were stimulated with 10 U/ml Epo for 3 or 24 h. Fig. 1 shows the effect of Epo on PTP1B expression analyzed by Real-Time RT-PCR (a) and Western blotting (b). Differentiated UT-7 cells showed higher PTP1B mRNA levels than undifferentiated control cells, regardless of the Epo treatment applied. PTP1B mRNA levels in differentiated UT-7 cells decreased after the starvation period but it increased fourfold after 24 h of Epo stimulation. On the other hand, TF-1 cells cultured with Epo showed higher PTP1B mRNA levels than those cultured with GM-CSF. Even though cells deprived of serum and growth factor ($t = 0$) showed PTP1B expression at lower mRNA levels than those cultured with GM-CSF, enzyme expression was recovered after 24 h of Epo stimulation.

Different patterns of PTP1B protein expression were observed. Both cell models cultured in the presence of Epo showed a band of 46 kDa of PTP1B, in addition to the two expected bands of 50 and 42 kDa. This 46 kDa PTP1B isoform was recognized by the antibody directed against its N-terminal extreme (PH01), but not by the antibody directed against its C-terminal extreme (PH02), thus suggesting that it arose from a C-terminal truncation. In order to rule out the possibility that the appearance of the 46 kDa isoform were associated to changes in cell viability, the MTT assay was performed. Results show that cell viability remained constant for either TF-1 or differentiated UT-7 cells (data not shown).

Epo induces PTP1B enzymatic activity in TF-1 and differentiated UT-7 cells

It has been shown that PTP1B cleavage has important functional consequences, such as the increase of its catalytic activity [10,29,30]. Therefore, PTP1B phosphatase activity was assessed in TF-1 cells and differentiated UT-7 cells. Levels were measured by the rate of pNPP hydrolysis and expressed as percentage of control values. Fig. 1c shows that Epo-cultured UT-7 cells, either differentiated (H) or Epo-stimulated for 24 h after cell starvation, had significantly higher PTP1B enzymatic activity than cultures exposed to the other treatments. Likewise, TF-1 cells showed higher phosphatase activity in cells permanently cultured with Epo or Epo-stimulated for 24 h after cell starvation.

The 50 kDa PTP1B isoform is tyrosine phosphorylated in differentiated UT-7 cells and in TF-1 cells

Although PTP1B exists as a 50 kDa transmembrane protein in the endoplasmic reticulum and it can be released to the cytosol as a 42 kDa isoform, two other bands of approximately 48 kDa and 46 kDa appear to represent different phosphorylation states of the cytosolic isoform or truncated forms of the transmembrane PTP1B [5,31,32]. On the other hand, the phosphorylation of PTP1B has been related to its enzymatic activity. Bandyopadhyay et al. [33] have shown that insulin stimulates the phosphorylation of this protein in three tyrosine residues, increasing its phosphatase activity.

In order to clarify tyrosine phosphorylation of PTP1B isoforms in TF-1 and differentiated UT-7 cells, both cell types were cultured during 18 h without FBS and growth factors and then stimulated with 10 U/ml Epo for short periods. Afterwards, cell lysates were immunoprecipitated with anti-PTP1B antibody followed by Western blot analysis with anti-PY antibody (Fig. 2a). We have already reported that Epo stimulates PTP1B tyrosine

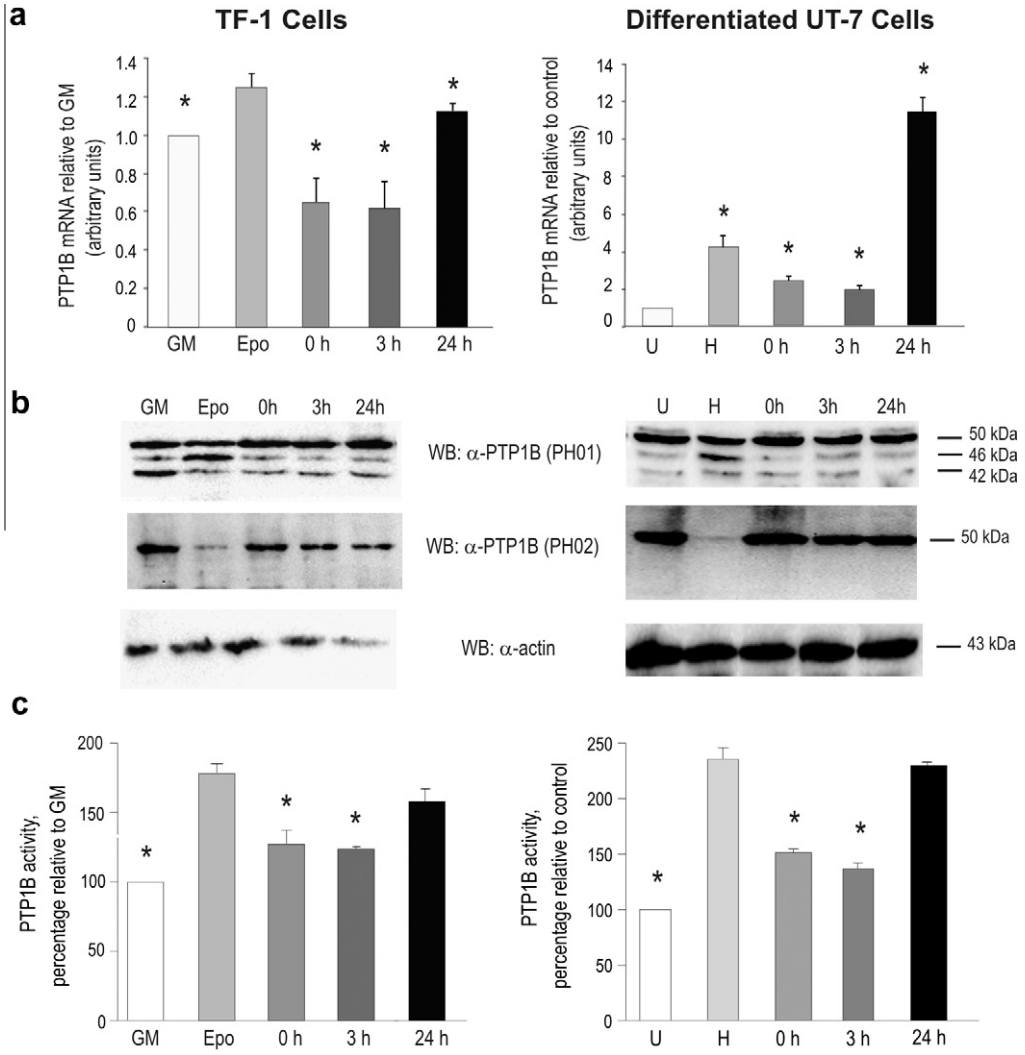

Fig. 1. PTP1B mRNA level, protein cleavage and enzymatic activity in TF-1 and differentiated UT-7 cells. TF-1 cells were cultured in medium without serum and growth factor. After 18 h-starvation (t = 0 h), cells were stimulated with 10 U/ml Epo during 3 or 24 h. Cells permanently cultured with GM-CSF (GM) or Epo were used as controls. UT-7 cells (1×10^7) were induced to erythroid differentiation with 30 μM hemin in IMDM, 10% FBS, and 2 U/ml Epo for 48 h. Differentiated UT-7 cells were cultured in medium without serum and Epo. After 18 h starvation (t = 0 h), cells were stimulated with 10 U/ml Epo during 3 or 24 h. Undifferentiated (U) and differentiated UT-7 cells (H) permanently cultured with 2 U/ml Epo were used as controls. (a) Total RNA was extracted and PTP1B expression quantified by Real-Time PCR. Results are reported as arbitrary units relative to GM-cultured TF-1 cells (GM) or to control undifferentiated UT-7 cells (U). (*) Significant differences with respect to Epo and U assays for TF-1 cells or to UT-7 cells, respectively ($P < 0.05$). (b) Cell lysates were prepared and resolved by SDS–PAGE. Proteins were transferred to NC membranes and detected by immunoblotting using anti-PTP1B antibodies (PH01 detects PTP1B N-terminal extreme and PH02 detects PTP1B C-terminal extreme), or anti-actin as loading control. (c) Total lysates were prepared and PTP1B was immunoprecipitated with anti-PTP1B antibody. PTP1B phosphatase activity was determined in immunoprecipitates by measuring optical density at 405 nm proportional to the product derived from the pNPP hydrolysis. Results are reported as percentage activity with respect to that of either GM cultured TF-1 cells or undifferentiated UT-7 cells. The figure shows representative patterns of three independent experiments. Each bar represents mean ± SEM (n = 3). (*) Significant differences with respect to Epo and H treatments for TF-1 cells or to UT-7 cells, respectively ($P < 0.05$).

phosphorylation in undifferentiated UT-7 cells [20]. However, in the present study we observed an opposite kinetic pattern in TF-1 and differentiated UT-7 cell cultures. In cells grown in the presence of Epo, phosphorylated PTP1B remained in this state after the starvation period. This process was reverted after Epo stimulation for 5 min. Tyrosine phosphorylation was reestablished after subsequent Epo stimulation for 60 min. The only isoform phosphorylated in both cell types was 50 kDa PTP1B.

The 46 kDa PTP1B isoform remains in the subcellular membrane fraction

It has already been shown that the 50 kDa PTP1B isoform is attached to the endoplasmic reticulum by its 35 amino acid C-terminal sequence [5] and that the 42 kDa isoform is located in the cytosol [29]. Then, we investigated the subcellular location of the 46 kDa isoform. For this purpose, TF-1 cells and differentiated UT-7 cells were treated as described above. The preparation of subcellular fractions allowed us to analyze membrane proteins by Western blotting. Fig. 2b shows that 46 kDa PTP1B remained in the membrane fraction of both TF-1 and differentiated UT-7 cells. Surprisingly, the 46 kDa band also appeared in the membrane fraction of both cell types after 24 h of Epo stimulation, although it was hardly observed in total protein Western blotting. These results were confirmed by western blots using PH02 antibody, in which PTP1B was hardly detected in membrane fractions of UT-7 cells, either differentiated (H) or Epo-stimulated for 24 h after cell starvation, and in TF-1 cells permanently cultured with Epo or Epo-stimulated for 24 h after cell starvation (not shown). This probably occurred because 46 kDa PTP1B was more concentrated among membrane proteins after subcellular fractionation, and thus it could be easily detected.

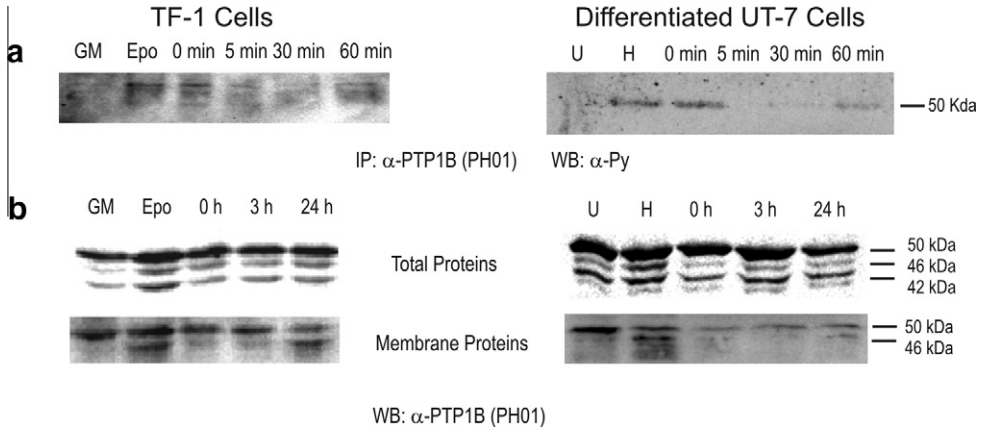

Fig. 2. PTP1B subcellular localization and tyrosine phosphorylation in TF-1 and differentiated UT-7 cells. (a) After serum and growth factor starvation, cells were stimulated with 10 U/ml Epo and after short periods of cell stimulation, PTP1B was immunoprecipitated with anti-PTP1B antibody from total lysates, and analyzed by Western blotting probed with anti-phosphotyrosine antibody (α-PY). (b) Cells (1×10^7) were cultured as described in Fig. 1. Cell lysates were subjected to subcellular fractionation and membrane proteins were resolved by SDS–PAGE, transferred to NC membranes and detected by immunoblotting using the anti-PTP1B antibody PH01. Blots are representative of three independent experiments with similar results.

Calpain is involved in Epo-induced PTP1B cleavage in TF-1 and differentiated UT-7 cells

It has been reported that the 42 kDa PTP1B isoform results from calpain-mediated cleavage in platelets [10,29,34] and in a breast cancer cell line [35]. To determine whether 46 kDa PTP1B is also a cleavage product induced by calpain, GM-stimulated TF-1 cells or undifferentiated UT-7 cells were pretreated with or without the calpain specific inhibitor calpeptin, and then incubated with the calcium ionophore A23187. A cell culture pretreated with EGTA and $MgCl_2$ was used as negative control. As shown in Fig. 3, a major 46 kDa band was detected when GM-cultured TF-1 cells or undifferentiated UT-7 control cells were treated with the calcium ionophore A23187 in the presence of extracellular calcium, in agreement with results observed in Epo-stimulated TF-1 cells or hemin-differentiated UT-7 cells. Co-incubation with the ionophore in the presence of calpeptin or EGTA/$MgCl_2$ abolished PTP1B cleavage partially in TF-1 cells and completely in differentiated UT-7 cells. The different isoforms were detected using antibodies directed against either C- or N-terminal extremes of PTP1B.

TF-1 cells and differentiated UT-7 cells show different calcium response to Epo

Calcium is one of the intracellular second messengers that influence many cell functions in erythroid cells through EpoR. Calcium has an important role in colony growth and in the terminal stages of cell differentiation [36–39]. On the other hand, calpain is a calcium-dependent cysteine protease. Thus, in order to find a relationship between Epo and calpain-induced PTP1B cleavage, we analyzed calcium influx in response to Epo stimulation in both cell models. Flow cytometry assays showed higher intracellular calcium content in differentiated UT-7 cells than in undifferentiated controls under basal conditions (cells permanently cultured with Epo) (Fig. 4a). The presence of Epo in TF-1 cells also increased basal intracellular calcium content with respect to TF-1 cells grown with GM-CSF (Fig 4b). On the other hand, Epo stimulation increased calcium concentration even after 60 min in TF-1 and differentiated UT-7 cells, whereas the calcium influx decreased and stopped after 30 min of Epo stimulation in UT-7 undifferentiated cells (Fig. 4c). These results agree with reports from Miller et al. [40], who demonstrated that the magnitude of changes in intracellular calcium in

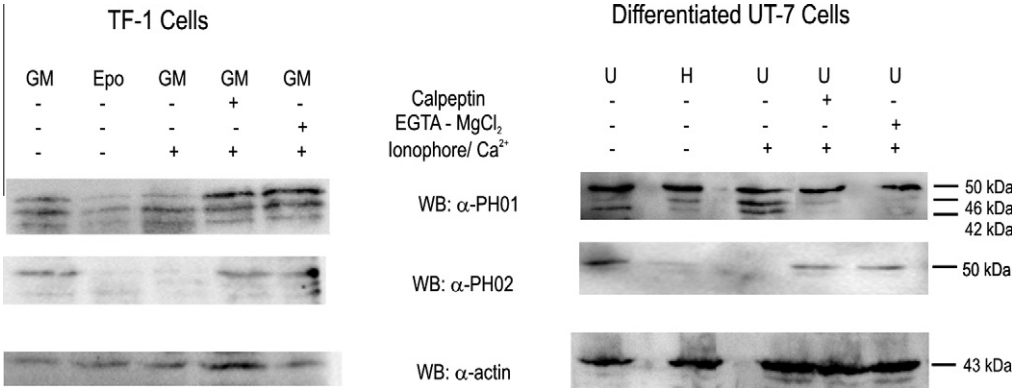

Fig. 3. Calpain-mediated cleavage of PTP1B in TF-1 and differentiated UT-7 cells. TF-1 cells (1×10^7) were cultured for 18 h with GM-CSF or Epo in RPMI medium-10% FBS. UT-7 cells (1×10^7) were induced to erythroid differentiation with 30 μM hemin for 48 h. Undifferentiated (U) and hemin-treated (H) UT-7 cells were cultured in IMDM in the presence of 2 U/ml Epo along the whole experience. Calcium influx was stimulated with 1 mM ionophore A23187 for 15 min in the presence of 1 mM $CaCl_2$. Calpeptin or EGTA pre-treatments were carried out by cell preincubation with either 20 mg/ml calpeptin or 1 mM EGTA plus 2 mM $MgCl_2$ for 30 min at room temperature, before the addition of A23187 and $CaCl_2$. PTP1B was detected in cell lysates by immunoblot analysis as described in Fig. 1. These results are representative of three independent experiments.

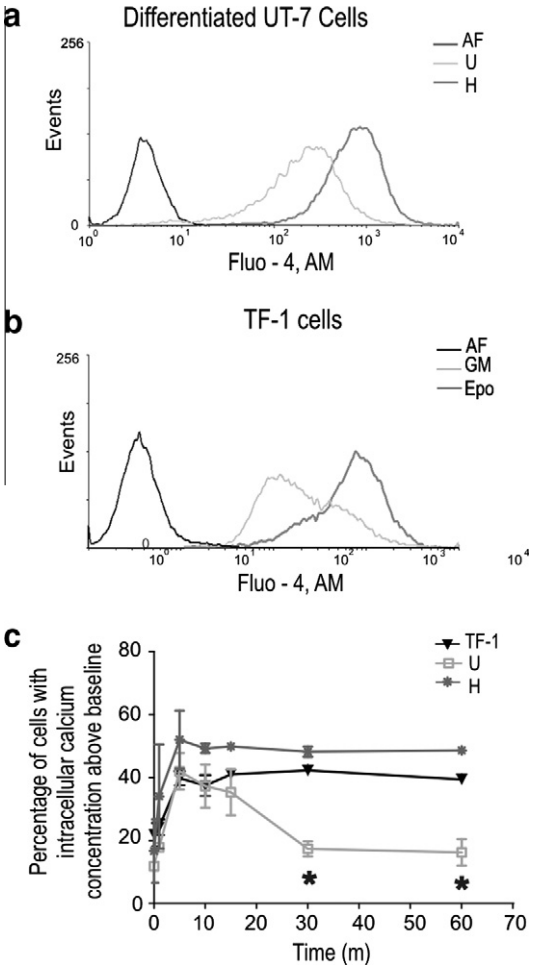

Fig. 4. Intracellular calcium content in TF-1 and differentiated UT-7 cells. Undifferentiated and differentiated Epo-cultured UT-7 cells (a) as well as TF-1 cells cultured with either GM-CSF or Epo (b) were loaded with Fluo-4, AM for 20 min in order to measure intracellular calcium content by flow cytometry. The figure shows the corresponding histogram profiles of intracellular calcium content. AF means autofluorescence of cells without Fluo-4, AM. (c) Cells (1×10^6) were starved from serum and growth factor for 18 h and, after short periods of stimulation with 10 U/ml Epo, calcium content was quantified by flow cytometry. Data were analyzed by the WinMDI software. The figure shows Epo modulation of intracellular calcium content in TF-1 as well as in undifferentiated and differentiated UT-7 cells. Results are reported as percentage of cells with calcium content above the baseline. Baselines were established as fluorescence determined in TF-1 cell cultures with GM-CSF or in UT-7 cells permanently cultured with Epo. Each point represents mean ± SEM ($n = 3$). (*) Significant differences with respect to TF-1 or differentiated UT-7 cells after 30 or 60 min of Epo stimulation ($P < 0.05$). Figures a and b show representative patterns of three independent experiments.

response to Epo is high in partially hemoglobinized and more mature erythroblasts and minimal in very immature erythroblasts.

TRPC3/ TRPC6 relationship is increased by Epo action

TRPC3 and TRPC6 are two calcium-permeable channels differentially expressed on human erythroid precursors. Reports confirmed that only TRPC3 activity is regulated by Epo [15,41]. Moreover, it has been demonstrated that TRPC6 expression decreases during erythroid differentiation along cell maturation from CD34+ cells into erythrocytes, resulting in a significant increase of the TRPC3/TRPC6 ratio. The rise of intracellular calcium concentration in response to Epo stimulation parallels the increase of TRPC3/TRPC6 ratio until late stages of erythroid differentiation when EpoR expression is reduced [16]. In order to determine TRPC3/TRPC6 relationship, TF-1 and differentiated UT-7 cells were starved and stimulated as described in the experiments above, and calcium channel protein expression was quantified by Western blotting and band densitometry. Fig. 5 shows that TRPC3/TRPC6 relationship was significantly higher in TF-1 cells cultured in the presence of Epo or stimulated with Epo for 24 h after 18 h-starvation. UT-7 differentiated cells cultured with Epo or Epo-stimulated for 24 h after starvation also showed a significantly higher TRPC3/TRPC6 ratio than cells used in the other treatments.

Discussion

PTP1B is a prototype protein tyrosine phosphatase that takes part in the regulation of several signaling pathways that involve tyrosine phosphorylation induced by growth factors, cytokines and hormones. It is also one of the phosphatases involved in Epo downregulation [42]. Since phosphorylation and dephosphorylation are key enzymatic reactions in the Epo signaling pathway, PTP1B is thoroughly regulated in erythroid growth and differentiation because these processes are Epo-dependent.

Different mechanisms of PTP1B modulation have been reported at the transcriptional, translational and post-translational levels [20,43,44]. However, little is known about PTP1B regulation in the Epo-EpoR signaling cascade. The present study describes a novel function of intracellular calcium-dependent protease calpain in the differential regulation of PTP1B among cell lines and erythroid differentiation stages. In a previous report, we demonstrated that Epo induced PTP1B expression, which was associated to increases in PTP1B tyrosine phosphorylation and enzymatic activity in the Epo-dependent UT-7 cell line [20]. Moreover, we found that Jak2 and PI3K were involved in this upregulation. In order to complete these previous results, the present study was focused on the assessment of PTP1B modulation in UT-7 cells at a later stage of erythroid differentiation, as well as in TF-1 cells, which do not strictly depend on Epo to survive.

PTP1B expression was measured in TF-1 and differentiated UT-7 cells cultured in the absence of growth factors and nutrients and subsequently stimulated with Epo (a similar treatment to the one applied to undifferentiated UT-7 cells in our previous study). Surprisingly, it was observed that although PTP1B mRNA decreased after cell starvation and returned to control levels after Epo-stimulation, PTP1B protein levels remained almost constant. This suggests that the starvation period was not long enough to reach PTP1B protein degradation, and thus PTP1B protein level up-modulation would not be necessary.

PTP1B has been described either as a 50 kDa protein attached to the endoplasmic reticulum or as a 42 kDa isoform located in the cytosol. However, in both TF-1 and differentiated UT-7 cells permanently grown with Epo or stimulated with Epo for 24 h after starvation, we found two cleaved PTP1B isoforms of 46 kDa and 42 kDa in addition to the expected 50 kDa isoform. Even though the three isoforms have already been described, 46 kDa PTP1B is the most unusual. Bhoola and Hammon [22] observed that the 46 kDa band was predominant in differentiated and undifferentiated granulocytic HL60 cells, but that dynamic temporal changes in PTP1B expression occurred when cell differentiation was induced.

To further study the 46 kDa isoform, we tried to determine its origin, considering that it could be either the result of the 42 kDa isoform phosphorylation or a cleavage product of the 50 kDa protein.

Bandyopadhyay et al. [33] demonstrated that insulin stimulates PTP1B phosphorylation in three tyrosine residues. The epidermal growth factor receptor also phosphorylated PTP1B N-terminus on a motif flanking Tyr-66 [45]. We have already observed that Epo induces PTP1B tyrosine phosphorylation in undifferentiated UT-7

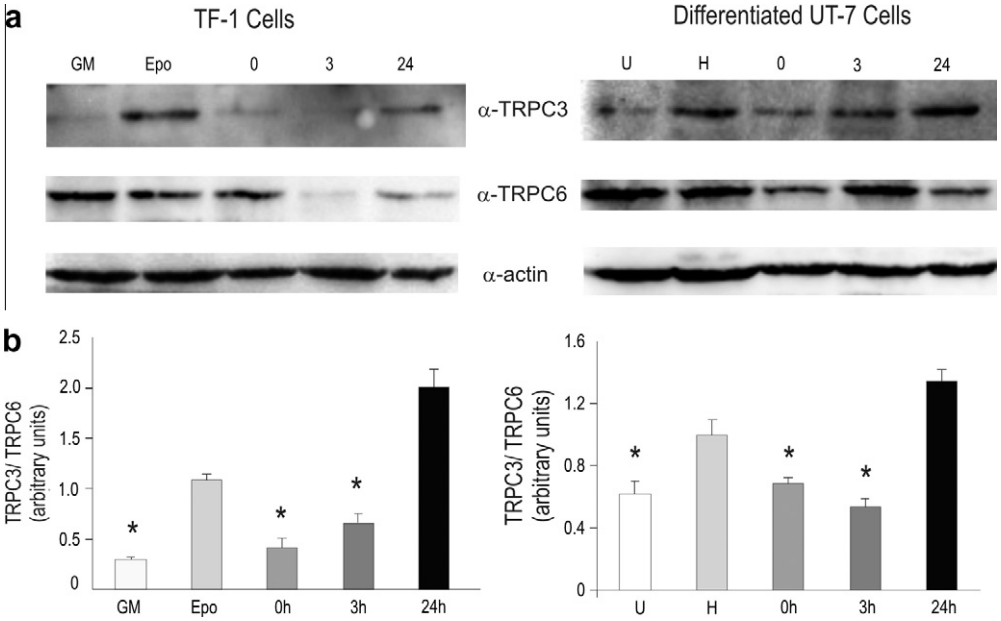

Fig. 5. TRPC3/TRPC6 relationship in TF-1 and differentiated UT-7 cells. Cells (1×10^7) were treated as described in Fig. 1. Cell lysates were prepared and resolved by SDS–PAGE. Proteins were transferred to NC membranes and detected by immunoblotting using anti-TRPC3, anti-TRPC6, or anti-actin as loading control. Densitometric analysis was performed to quantify the TRPC3/TRPC6 relationship (b). Each bar represents mean ± SEM ($n = 3$). (*) Significant differences with respect to Epo for TF-1 cells or to H for UT-7 cells ($P < 0.05$). Figure shows representative patterns of three independent experiments.

cells [20]. Conversely, in TF-1 and differentiated UT-7 cells Epo induced PTP1B dephosphorylation. Moreover, the only phosphorylated isoform is 50 kDa PTP1B, suggesting that Epo could only induce the phosphorylation of a tyrosine residue located in PTP1B C-terminus. Based on these results, it seems unlikely that the 46 kDa PTP1B isoform derived from 42 kDa phosphorylation.

Therefore, the location of the 46 kDa isoform was determined after subcellular fractionation. As in the case of 50 kDa PTP1B, the 46 kDa isoform was found in the heavy membrane fraction enriched with endoplasmic reticulum (ER) and plasmatic membrane [28]. This result is in agreement with previous reports which have demonstrated that complex formation of PTP1B with plasma membrane anchored proteins is possible without detachment of PTP1B from the ER and that the dynamic ER membrane network is in constant contact to the plasma membrane [46,47]. Assays using calcium ionophore A23187 and calpain specific inhibitor calpeptin showed that 46 kDa PTP1B results from calpain-mediated cleavage. Its appearance coincided with an increase of PTP1B enzymatic activity. These results agree with reports from Frangioni et al. [29], who observed that PTP1B calpain-catalyzed cleavage was associated with 2-fold enzymatic activity stimulation in platelets. However, the subcellular location of the 46 kDa band is not in the cytosol as in the case of the 42 kDa isoform. It is worth mentioning that Cortesio et al. [35] also demonstrated that PTP1B have an increased enzymatic activity when it is cleaved by calpains.

Calpains are calcium-dependent thiol proteases expressed in mammalian cells. On the other hand, Epo has been shown to induce an increase in intracellular free calcium [12,13], a universal intracellular second messenger that influences many cell functions [39]. Some authors have suggested that the regulation of intracellular calcium mediated by Epo is one of the signaling mechanisms controlling the proliferation and differentiation of erythroid cells [12,13,36,46]. It has been reported that Epo induced an increase in intracellular calcium in late and not in early human erythroid precursors, suggesting that changes in intracellular calcium concentrations may be related to cell differentiation rather than proliferation [40]. In accordance with these results, we found that differentiated UT-7 cells had a more sustained calcium influx upon Epo stimulation than undifferentiated cells, and that TF-1 cells showed similar behavior to differentiated UT-7 cells.

Signal transduction mediated by Epo involves a series of biochemical and ionic regulatory events. According to Cheung et al. [12], erythropoietin modulates a voltage-independent calcium channel in human erythroblasts. The opening of this calcium channel rather than the intracellular calcium mobilization by IP3 virtually accounts for the increase of calcium in Epo-treated erythroblasts. Recent studies demonstrated that the transient receptor potential channel TRPC3 is the Epo-regulated calcium channel in human erythroid cells, and that it acts through a mechanism that involves the activation of PLCγ and the interaction of TRPC3 with PLCγ and IP3R. It has also been found that TRPC3 expression increases whereas TRPC6 expression decreases during erythroid differentiation. This can be associated with a dramatic increase of intracellular calcium in response to Epo stimulation, suggesting that the TRPC3/TRPC6 ratio is physiologically significant during erythroid differentiation [16]. Consequently, we studied TRPC3 and TRPC6 expression and obtain similar results to those previously reported, suggesting that the higher the TRPC3/TRPC6 ratio, the higher the calcium response to Epo. TRPC6 could modulate TRPC3 activity through the inhibition of the Epo-stimulated increase of TRPC3 expression in the cell surface, but this effect may change due to the modulation of channel expression by cell differentiation. This could account for the low TRPC3 expression detected in undifferentiated UT-7 cells, which would led to poor calcium influx in response to Epo.

Conclusions

Our findings in Epo-stimulated cultures of TF-1 cells and differentiated UT-7 cells suggest that there is a similar pattern of PTP1B modulation related to increased TRPC3/TRPC6 expression ratio which could consequently lead to higher and sustained calcium response to Epo. As a consequence, calcium-activated calpain modulates PTP1B cleavage leading to increased phosphatase activity. Therefore, our results strongly suggest that the route

Epo-calpain-PTP1B plays a key physiological role during cellular erythroid differentiation.

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