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Original Communication

RT-PCR analysis of the expression of the cytoplasmic protein tyrosine phosphatases Tc-PTP (*PTPN2*) and PTP1B (*PTPN1*), in cultivated splenic lymphocytes from *Sus scrofa*

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ABSTRACT

In the present study, the expression of two non-receptor protein tyrosine phosphatases in pig spleen lymphocytes (splenocytes) was analyzed. The mRNA-transcripts of PTP1B and Tc-PTP were analyzed by reverse transcriptase-PCR in cultured splenic lymphocytes exposed to different biochemical treatments. Mitogenic stimulation by concanavalin A produced a clear increase in the mRNA relative amounts of both PTPs, after 24-h exposure in primary cultures, and LPS exerted a marked increase of both splice variants of the Tc-PTP (ER and nuclear). Interestingly, both PTPs seem to respond to the redox status, given that the Tc-PTP-mRNA showed a marked increase in the presence of 1.9 mM H_2O_2, and PTP1B showed a similar effect after exposure of the cultured splenocytes to 10 mM N-acetylcysteine for 24 h. Other biochemical treatments of the cultured splenocytes also showed an opposite effect in the mRNA-levels of these PTPs: lowering of the Tc-PTP-mRNA and increase of the PTP1B transcript after exposure to IL-4 and γIFN. The results suggest non-redundant functions of both PTPs, despite their similar structures. The Tc-PTP showed an increase in the mRNA level after H_2O_2 treatment of the splenic lymphocytes for a short time (1.5 h). The results suggest that the intracellular redox status may play an important role in the regulation of the expression of PTP1B and Tc-PTP in splenocytes.

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KEYWORDS: Tc-PTP, PTP1B, signaling, tyrosine phosphorylation, mRNA transcripts, cytokines, redox

ABBREVIATIONS

ConA	:	concanavalin A
NAC	:	N-acetylcysteine
αIg-AB	:	anti immunoglobulin antibodies
IL	:	interleukin
LPS	:	lipo-polysaccharide
FCS	:	fetal calf serum
GapDH	:	glyceraldehyde 3-P dehydrogenase
IFN	:	interferon
PTP	:	protein tyrosine phosphatase
PTK	:	protein tyrosine kinase
DEPC	:	diethyl pyrocarbonate
ME	:	mercaptoethanol
ER	:	endoplasmic reticulum

INTRODUCTION

Protein tyrosine phosphatases (PTPs) constitute a large family of enzymes with great structural diversity (for review: Tonks, 2006 [1]). PTPs regulate signal transduction by controlling tyrosine phosphorylation in fundamental cellular functions such as growth, proliferation, migration and apoptosis [2-5]. The PTP superfamily genes encode 'classic' pTyr phosphatases (PTPs) and 'dual specific' phosphatases (DSPs) [6]. PTPs were originally considered as 'housekeeping enzymes', but later research showed that these enzymes are key elements in the regulation of signal transduction in different physiological systems and pathways, offering new therapeutic

targets under pathological threats [7-9]. PTPs include approximately 100 genes of structurally and functional diverse enzymes (there are about 90 human pTyr-kinase (PTK) genes with similar complexity in structures and regulation). The PTP family can be divided into the so-called receptor and non-receptor forms, which possess a catalytic and a regulatory domain that docks the enzymes to subcellular structures.

The first-described member of the PTP family was PTP1B [10, 11]. PTP1B (gene symbol: *PTPN1*) is the prototype of the family of 'soluble' PTPs [12]. The enzyme has a catalytic domain at the N-terminus, with two proline-rich motifs in tandem position at the regulatory, CT domain (human PTP1B), which allows it to interact with SH3 domains [13, 14]. This PTP is ubiquitously expressed in vertebrates. The 35 terminal residues of the human-PTP1B are predominantly hydrophobic and anchor the enzyme to the cytoplasmic side of the ER membrane [15]. Despite its broad distribution in lymphoid cells, the participation of PTP1B in the immune response has not been clearly defined [16]. Tc-PTP is a 'cytoplasmatic' protein Tyr-phosphatase, showing 72% identity with PTP1B in the catalytic domain (amino acids 43-288 of the human Tc-PTP sequence), as well as high similarities in their tertiary structures [17, 18]. Originally, Tc-PTP (gene symbol: *PTPN2*) was cloned from a cDNA library of human peripheral T cells [19, 20]. Tc-PTP shows splicing variants of 48 kDa (bound by a hydrophobic end of its CT to the ER membrane), and a 45 kDa form, which contains two nuclear localization signals for reversible anchoring to nuclear structures [21].

Tyr phosphorylation is one of the most relevant molecular mechanisms in signaling cascades in immune cells [22, 23]. It participates in the early activation of the immunoreceptor tyrosine-based activation motifs (ITAM) of the T cell receptor [24], the activatory phosphorylations of the ITAMs of the B cell receptor (BCR) in naive B cells, and the cytokine activated pathways in different tissues [25], where the Tyr-phosphatases are known to play crucial, positive and/or negative roles [26]. The role of PTPs 1B and Tc- in the immune response seems to be different and non-redundant. There are results showing that both enzymes would act in a complementary form or could as well perform different/opposite roles. *In vitro* studies with cell cultures showed that PTP1B and Tc-PTP control early cytokine-signaling events in the immune response inhibiting the Jak/STAT pathway [27]. PTP1B would dephosphorylate the Janus Tyr-kinases Jak2 [28] and Tyk2 [29], whereas Tc-PTP acts negatively (dephosphorylating activatory P-Tyrs) on Jak1 and Jak3 [30]. 'Substrate-trapping' mutants of PTP1B and Tc-PTP allowed the identification of possible substrates of both PTPs, showing that STAT5a/b could be immunoprecipitated by inactive mutants of both PTPs [31], but STAT1 and STAT3 would be physiological substrates of the Tc-PTP [32]. Thus, there is experimental evidence that both Tc-PTP and PTP1B are essential elements in the control of cytokine signaling pathways in the immune system and are involved in the normal development of hematopoietic cell lines [33, 34]. According to Mustelin *et al.* [16], the PTPs CD45 (*PTPRC*) and PTP1D (SHP-2; gene *PTPN11*) exert a positive effect leading to T cell early activatory events, and Tc-PTP and PTP1C (SHP-1; gene *PTPN6*) play a negative role by controlling different signaling events of the activation of T lymphocytes; PTP1B would not be involved in such signaling processes [16].

In the present study the expression of PTPs Tc-/1B- were examined in lymphocytes obtained from the spleen of the domestic pig, to determine whether and how these two structurally homologous enzymes respond to cytokines (IL-4, IL-6 and γIFN) and mitogenic stimuli (ConA, LPS), and if both PTPs could be controlled at their expression level by redox molecules in cultured immune cells. The study contributes to the understanding of the expression events (transcription/transcripts half-life) of both non-receptor and structurally related PTPs during activation of splenic lymphocytes, functioning as feasible positive/negative regulators of the immune response.

MATERIALS AND METHODS

Chemicals

Salts, buffers and biochemical reagents were all of the highest purity available. Water was purified with a Milli-Q Ultrapure (Millipore) device, with UV_{254}–treatment. Molecular biology reagents were from Roche and MBI-Fermentas; PCR primers

were from MWG-Biotech; cytokines were from Biomol and Transduction Laboratories.

RNA extraction

For the preparation of mRNA, DEPC-treated water was used. Total RNA was prepared according to Chomczynski and Sacchi [35], and commercial kits for the isolation of total RNA were also used (Qiagen). 20-30 mg of cultured splenocytes were removed from the culture flasks/dishes, placed in polypropylene tubes (phenol/chloroform resistant), the culture media was removed and RNA was extracted in 2 ml of the buffer (pH 7.0): 4 M guanidinium isothiocyanate, 0.5% sarkosyl, 0.1 M β-ME, 25 mM sodium citrate. A Polytron homogenizer was utilized, with 3 cycles each consisting of 30 seconds homogenization/30 seconds pause (at 4 °C). Following solutions were added: 0.1 ml of 2 M NaAc (pH 4.0), 1 ml of water-saturated acidic phenol, and 0.2 ml of chloroform/isoamyl alcohol (49:1). This extraction mixture was incubated on ice for 15 min and then centrifuged (20,000 g for 20 min, 4 °C). The aqueous supernatant was carefully taken and put into Eppendorf tubes of 1.5 ml. 1 ml of ice-cold isopropanol was added and the RNA was precipitated at -20 °C for 60-90 min. Total RNA was obtained by centrifugation (13,000 rpm, 25 min, 4 °C). RNA was resuspended by adding 0.3 ml of RNA extraction solution and then 0.3 ml isopropanol was added to precipitate RNA again (60 min, -20 °C). The RNA pellet obtained by centrifugation was washed with 0.3 ml of ice cold 70% ethanol and centrifuged (10 min, 4 °C, 13,000 rpm). The total RNA pellet was finally resuspended in DEPC-treated water for amplification by RT-PCR. The RNA was quantified and used immediately for amplifications.

RNA quantification

Total RNA was quantified spectrophotometrically at 260 nm, and the purity was determined according to the ratio A_{260}/A_{280}. Total RNA quality was also assessed in agarose-formaldehyde gels in the presence of ethidium bromide.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from cultivated pig splenic lymphocytes was immediately utilized for RT-PCR analysis. Enzymes and nucleotides for RT-PCR were from Roche. cDNA synthesis proceeded as follows: 2 µg total RNA (in 10 µl DEPC-water) was incubated for 10 min (at 65 °C) in the presence of 40 A_{260} mUnits of dT_{15} primer, and then cooled immediately on ice. The reaction mixture for the RT enzyme (in a final volume of 20 µl of RT-buffered solution) contained: 0.2 mM dNTPs, 25 U RNase inhibitor, and 40 U reverse transcriptase (0.8 µl; AMV-RT, Roche). The cDNA-synthesis mixtures were incubated for 20 min at 40 °C, 20 min at 42 °C, 15 min at 45 °C, and 10 min at 50 °C. Finally, the tubes were placed on ice.

The cDNA was extracted with phenol/chloroform and precipitated with ethanol before the PCR amplifications. Resuspended cDNA was amplified by PCR, in 100 µl individual reactions. Each PCR tube contained 1x buffer (Roche), 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.01 pmol/µl of 5' and 3' primers (final concentrations), and 2 µl of cDNA. PCR amplifications were as follows: 5 min at 94 °C, 80 °C (pause); 1.5 U Taq DNA polymerase (Roche) was then added to each tube. 34 amplification cycles were applied, and each cycle consisted of heating for 1 min at 94 °C, then 30 sec at 54 °C, and 1 min at a polymerization temperature of 72 °C. After the 34 cycles, the amplification mixtures were maintained for 4 min at 74 °C, and then cooled to 4 °C. Samples were used immediately for agarose electrophoresis or stored at -20 °C.

The amplification primers used were:

Tc-PTP (*PTPN2*):
ER variant, according to [19]; nuclear form of Tc-PTP [36]

Tc-NT 5'-ATGCCCACCACCATCGAGCGG-3' (1 → 21)
Tc-CT 5'-TTATAGGGCATTTTGCTGAAAAAAC-3' (1224 ← 1248)
Tc-Nuc 5'-TTAGGTGTCTGTCAATCTTGGCCT-3' (1141 ← 1164)

PTP1B (*PTPN1*): according to [12]
1B-NT 5'-CAAGTCCGGGAGCTGGGCGGCCATT-3' (33 → 57)
1B-CT 5'-GTTGAACAGGAACCTGTAGCAGAGGTA-3' (1270 ← 1296)

GapDH: (NCBI-Code: AF 261085.1)
Gap-5' 5'-CAAAAGGGTCATCATCTCTGCCCC-3' (348 → 371)
Gap-3' 5'-CCTGCTTCACCACCTTCTTGATGT-3' (770 ← 793)

Amplified DNAs corresponding to Tc-PTP, PTP1B and GapDH were tested for specificity cutting with restriction endonucleases and were also cloned in pBL-SK(+) and sequenced.

DNA electrophoresis was performed in 1% agarose gels in TAE-buffer (pH 8.0), consisting of: 40 mM Tris-HCl, 20 mM acetic acid, and 2 mM EDTA [37]. Electrophoresis in agarose gels (in the presence of ethydium bromide, at a final concentration of 0.3 μg/ml), was performed as described in Sambrook *et al.* [37]. Sample buffer for DNA (pH 7.8) was: 50 mM Tris-HCl, 20% glycerol, 0.4% bromophenol blue (BPB), and 2 mM EDTA. Equal volumes of the PCR-samples were placed on the DNA gels (run at 100 V, 60 min). Photos were taken under UV light (254 nm; Polaroid 667).

DNA standards (MBI-Fermentas) were:

- Marker "X"; λDNA/Eco91I (8454, 7242, 6369, 5686, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702, 224, 117 bp).
- Marker "M"; λDNA/EcoRI + HindIII (21227, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 125 bp).

Preparation of splenocytes from pig spleen

1-2 spleens from freshly slaughtered domestic pigs were transported to the laboratory in 1 liter beaker in sterile PBS (300 ml). Under sterile conditions the connective tissue was removed in a laminar flow chamber and the tissue was cut into 1 g pieces. These spleen pieces were pressed mechanically through a stainless steel sieve with 0.2 mm mesh size, and the splenic cells were collected in sterile PBS in a Petri dish. The suspension thus obtained was filtered through sterile cotton to capture connective tissues, macrophages and polymorphonuclear cells. The splenocytes thus obtained were centrifuged in sterile 50 ml Falcon tubes (500 g, 10 min, room temp.) and washed in 1x PBS. The splenic lymphocytes were then incubated with erythrocyte lysis buffer (pH 7.2), which consisted of: 0.1 M Tris-HCl, and 150 mM NH_4Cl.

The suspension was incubated at 30 °C for 15 min. It was shaken occasionally to keep the cells in suspension. The erythrocyte lysis was stopped by adding ice-cold 1x PBS solution and the cells were centrifuged (350 g, 10 min, room temp). These cells were washed twice in PBS. The cells obtained this way were resuspended in RPMI culture medium, as described below.

Cells were counted in a Neubauer chamber. Splenocytes were cultured in a density of 2 x 10^7 cells/ml, in sterile plastic culture dishes and incubated in a Heraeus cell oven (37 °C, 5% CO_2 atmosphere). These primary cultures were used then for pharmacological treatments before RT-PCR analysis.

Primary cell cultures

The lymphocytes were maintained in culture medium (RPMI-1640 + 10% FCS) with addition of antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and incubated at 37 °C under 5% CO_2 in culture plates of 5 cm diameter. FCS was initially heat-inactivated at 56 °C.

Cultured splenic lymphocytes were cultivated after preparation for 24 h in the above described culture medium, and then exposed for further 24 h (or the indicated period) to the mitogens/cytokines/redox chemicals as described in the 'Results' section.

Stimulation of immune cells

The cultivated splenocytes were treated with different chemicals or biochemical substances (mitogens, cytokines, oxidants, antioxidants; concentrations are indicated in the legends to figures). After each experiment, the treated cells were harvested gently with a micropipette and centrifuged, and the expression of the PTPs Tc-PTP and PTP1B was investigated by RT-PCR.

Software

Densitometric analysis of the relative amounts of the amplified DNAs was performed using the software ScionImage for Windows (compilation of NIH Image by Scion Corp.).

RESULTS

Amplification and cloning of protein tyrosine phosphatases of the domestic pig

In the present study the expression of two structurally related Tyr-phosphatases was analyzed after total RNA isolation and reverse transcriptase-PCR amplification. The expression of both PTPs was investigated in primary culture of splenocytes exposed to mitogenic stimulation by increasing concentrations of concanavalin A, for 24 h. Fig. 1 shows a positive amplification of a single transcript of the Tc-PTP

(ER form). The transcript shows a clear increment at higher concentrations of ConA, reaching an apparent relative maximal concentration after 24-h exposure to 10 μg/ml ConA. Similar results can also be observed for PTP1B in the splenic lymphocytes (Fig. 1). Control condition corresponded to 24 h of primary culture of pig splenocytes in culture medium (RPMI-medium, 10% FCS, antibiotics). As amplification control, a sequence of the GapDH enzyme was analyzed by RT-PCR (utilizing the same cDNA preparation as for the phosphatases), which showed no alteration in the presence of increasing concentrations of ConA (data not shown). Both amplified PTP transcripts correspond in size to the expected sequence lengths of ca. 1.2 kbp, in accordance with the respective human PTPs. The specificity of the amplified DNAs was monitored by cutting with restriction endonucleases, and both DNA-sequences were cloned and sequenced [38]. The accession codes of the Genbank for both pig sequences are EU753191 (Tc-PTP) and EU753192 (PTP1B).

Expression analysis of PTPs in cultivated splenocytes

Splenocytes from porcine spleen were isolated and cultivated for 24 hours (RPMI medium with addition of 10% FCS, at a culture density of 2×10^7 cells/ml). The splenocytes were further cultivated in three 5-ml culture plates for 15 hours with LPS (20 μg/ml) and in presence of H_2O_2 (1.7 mM); as the control condition, an untreated culture plate (all plates with 10% FCS) was incubated in parallel. While at the control condition (without stimulation), near to no amplification of the Tc-PTP could be observed (either the ER or the nuclear variants of the enzyme; Fig. 2, a, b), LPS produced a clear increase of both transcripts of Tc-PTP (Fig. 2, c, d). H_2O_2 produced also a clear increase in the expression of the Tc-PTP (both splicing variants; Fig. 2, e, f) after 15 hours of exposure of the cultured splenocytes. In parallel to the amplification of Tc-PTP, GapDH was amplified as control of constant total mRNA amounts in splenocytes cultivated in the presence and absence of hydrogen peroxide (Fig. 2, lanes g and h). The results indicate a possible redox control of the expression (and/or transcript stability) of the Tc-PTP.

In Fig. 2, the effect of hydrogen peroxide increasing the amount of Tc-PTP transcripts (ER and nuclear forms) can be clearly observed. Therefore, this effect was also investigated for PTP1B, as well as the effect on the expression of both PTPs in cultured splenocytes when exposed to the antioxidant NAC.

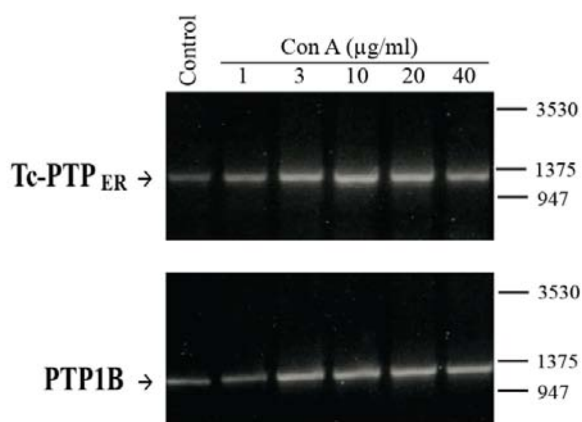

Fig. 1. RT-PCR analysis for the expression of Tc-PTP and PTP1B in the cultured splenocytes. Cells were extracted and cultured for 24 h (RPMI-10% FCS); then the splenocytes were stimulated for additional 24 h in the presence of the indicated concentrations of ConA (in μg/ml). As control of total mRNA-amounts, the expression of the enzyme GapDH was analyzed (data not shown). Primers for Tc-PTP were: Tc-NT-5' + Tc-CT-3'.

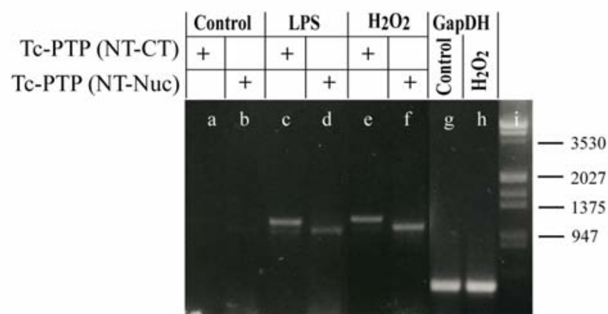

Fig. 2. RT-PCR amplification of cDNA sequences from pig spleen lymphocytes, after stimulation of cultured splenocytes. Cells were cultivated for 24 h (RPMI-10% FCS); then the cells were further cultivated for 15 h without stimulation (control, lanes a-b; g) and with addition of 20 μg/ml LPS (lanes c-d) or in the presence of 1.7 mM H_2O_2 (lanes e-f; h). RT-PCR from spleen total mRNA is also shown for the enzyme GapDH (lanes g-h). PCR was performed with the following primers for Tc-PTP: Tc-PTP_{ER} (Tc-NT-5' + Tc-CT-3'); Tc-PTP_{Nuc} (Tc-NT-5' + Tc-Nuc-3'). 2 μl cDNA (prepared using dT_{15}) was used for each PCR amplification.

Fig. 3 shows a marked activation of the expression of the Tc-PTP in the presence of H_2O_2 (3.7 mM, 24 h), without affecting the expression of the PTP1B, but in the presence of NAC (10 mM, 24-h exposure in the primary culture of splenocytes), the result was opposite: a clear increase in the PTP1B transcript was observed (Fig. 3, lane b). This result could mean a physiological control mechanism at the expression level of these PTPs effected by the redox status of the lymphocyte's environment. The expression of Tc-PTP was rapidly stimulated in the presence of 1.9 mM hydrogen peroxide, with a detectable increase after 90-min exposure of the cultured splenocytes to H_2O_2 (Fig. 4, A, lane b). Control '0 h' in Fig. 4 (B) corresponds to another culture plate of splenocytes in culture medium (RPMI-medium, 10% FCS, antibiotics), maintained for 5.5 h in the absence of H_2O_2 at the same culture conditions.

As further analysis, the effect of biochemical stimuli (mitogens, cytokines) on the expression of Tc-PTP and PTP1B in primary cultures of pig splenocytes was investigated. Fig. 5 indicates the different relative transcript amounts of the enzymes Tc-PTP (ER form), PTP1B and GapDH after 24-h exposure to the indicated molecules. An increase can be seen in the mRNA amount of the Tc-PTP after exposure to LPS for 24 h (10 and 20 μg/ml), in relation to the control condition; opposingly, PTP1B showed a diminishing transcript level after LPS treatment of the cultured splenocytes (Fig. 5, lanes b and c). ConA (5 μg/ml; 24-h exposure) exerted a similar activatory effect on the relative transcript amounts of Tc-PTP and PTP1B as shown in Fig. 1. On the other hand, IL-4 and γIFN produced similar effects on the transcript levels of both PTPs; both cytokines produced a marked lowering of the amount of Tc-PTP after 24-h exposure (specially with IL-4) and a clear increase of PTP1B transcript (in particular after γIFN exposure). Anti-Ig antibodies produced an increase of the transcript of PTP1B in the cultured splenocytes (Fig. 5, lane g), without an apparent change in the amount of the Tc-PTP transcript, and IL-6 (100 U/ml) exerted a similar increase in the levels of both transcripts (Fig. 5, lane f). The highest amounts of PTP1B transcripts were obtained after increasing the concentrations of NAC, with a maximum relative mRNA level for PTP1B obtained after 24-h exposure to 1.5 mM NAC (differing from the result obtained for the Tc-PTP). Thus,

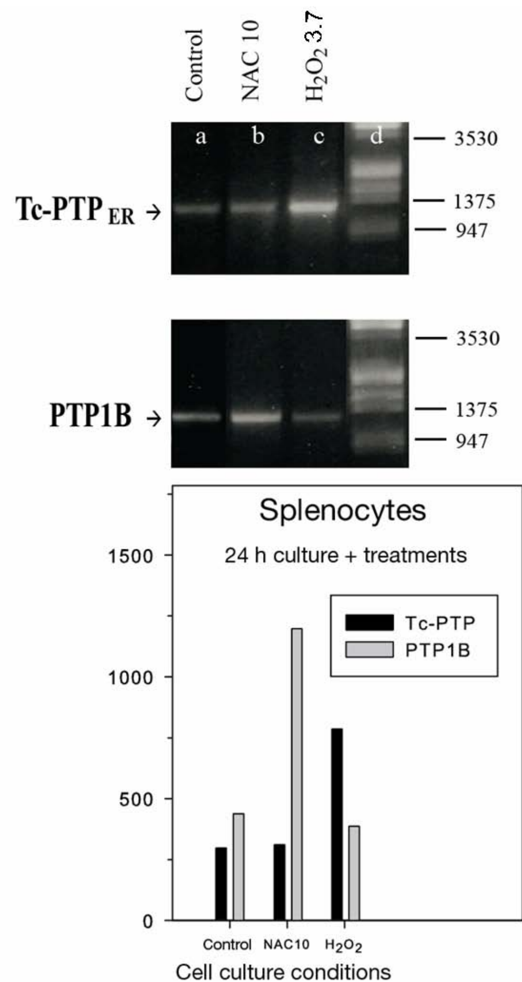

Fig. 3. RT-PCR analysis for the expression of Tc-PTP and PTP1B in cultivated splenocytes. Cells were extracted and cultured for 24 h; the splenocytes were exposed to further 24 h in the presence of 10 mM NAC (lane b) and 3.7 mM H_2O_2 (lane c); control plate corresponded to untreated cells during the 24-h culture (RPMI-10% FCS). Densitometric analysis for the relative amounts of amplified DNA of both PTPs.

the biochemical treatments for which both PTPs showed similar results were exposure to ConA and IL-6, and the cytokines which produced clear opposite results in the amounts of both PTP transcripts, were IL-4 and γIFN. Fig. 5 also shows that the expression level (and/or the half-life of the transcript) of the glycolytic enzyme GapDH apparently remained stable in the presence of all tested stimuli. It can be concluded that the gene expression of both PTPs (and/or the physiological control of the half-life of their mature transcripts)

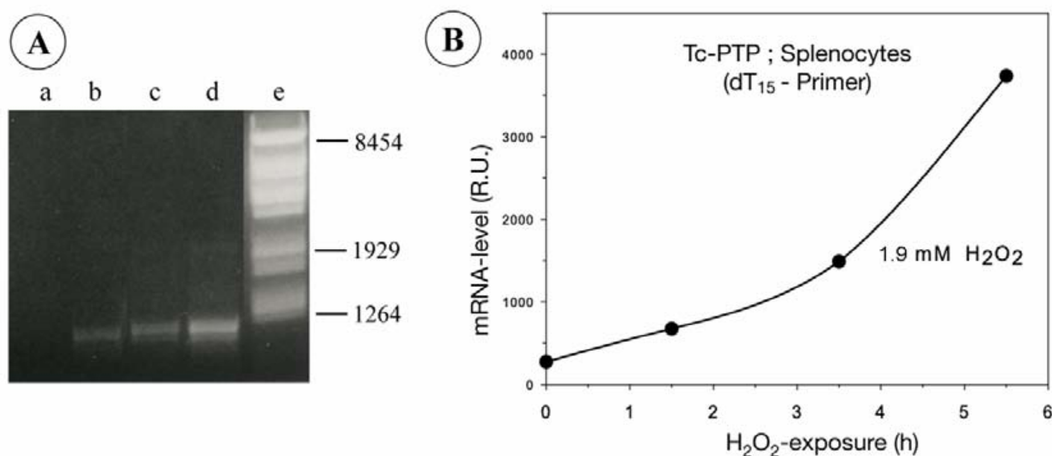

Fig. 4. RT-PCR analysis of the expression of Tc-PTP after H_2O_2 exposure in cultured splenocytes (4 different culture plates). (A) The RT-reaction was carried out with dT_{15} primer. The Tc-PTP primers were: Tc-NT-5' + Tc-CT-3'. Control (unstimulated cells; lane a); exposure to 1.9 mM H_2O_2, for 1.5 (lane b), 3.5 (lane c) and 5.5 h (lane d). (B) Densitometric analysis indicates the relative amount of the amplified DNA, in relative units (RU).

varies greatly, and that these fluctuations in response to exposure to different biochemical stimuli could be of physiological relevance during the control mechanisms of lymphocyte activation. Also, an opposite result in the regulation of mRNA amounts of Tc-PTP and PTP1B was observed in the cultured splencoytes.

DISCUSSION

The pig is a commonly used experimental organism because of its relatively high immunological similarity with the human, for the availability of desirable amounts of biological material (circulating cells/immune tissues), and for the low biological risks in relation to the handling of human tissues/fluids [39, 40]. It is used as a donor organism for xeno-transplants [41]. In the present study, lymphocytes of the pig spleen were isolated and cultivated to analyze the expression at mRNA level of two cytoplasmic, non-receptor protein tyrosine phosphatases. The PTPs 1B- and Tc- are structurally closely related enzymes that have a high degree of homology in their catalytic domains, but their biological functions, substrate specificity and the regulatory mechanisms in cells of the immune system seem to differ. The biological activity of members of the PTP super-family is controlled physiologically, among other mechanisms, through reversible phosphorylation, directed proteolysis, alternative splicing, differential sub-cellular localization, oxidation of catalytic amino acids and molecular associations with regulatory proteins [1, 16, 42]. Nevertheless, there is less detailed information about the regulation of the gene expression of structurally related PTPs (Tc-PTP/PTP1B or SHP-1/SHP-2) in different cells of the immune system (either at the transcriptional or translational level). In the present study, pig spleen lymphocytes were utilized. Splenocytes consist primarily of B- and T-lymphocytes [43, 44]; the adherent cells are retained by the cotton filter during the isolation of the splenic cells [43, 45]. In this procedure, a low amount of other cells (1-2% of monocytes, granulocytes) should be obtained from spleen.

To analyze the physiological role of PTPs 1B and Tc-PTP, null-mutant mice for these genes were generated [33, 46]. These experimental animals missing one of both PTPs had different phenotypes, which indicate different functions of these enzymes. Absence of Tc-PTP causes defects in hematopoiesis as well as changes in the immune response and the life expectancy was reduced [47]; PTP1B-deficient mice exhibited increased sensitivity to insulin, leptin and growth hormone [27, 48]. These results suggest that PTP1B and Tc-PTP constitute non-redundant enzymes. Different experimental results showed that PTP1B would be functionally associated with insulin receptor-induced intracellular events [48, 49].

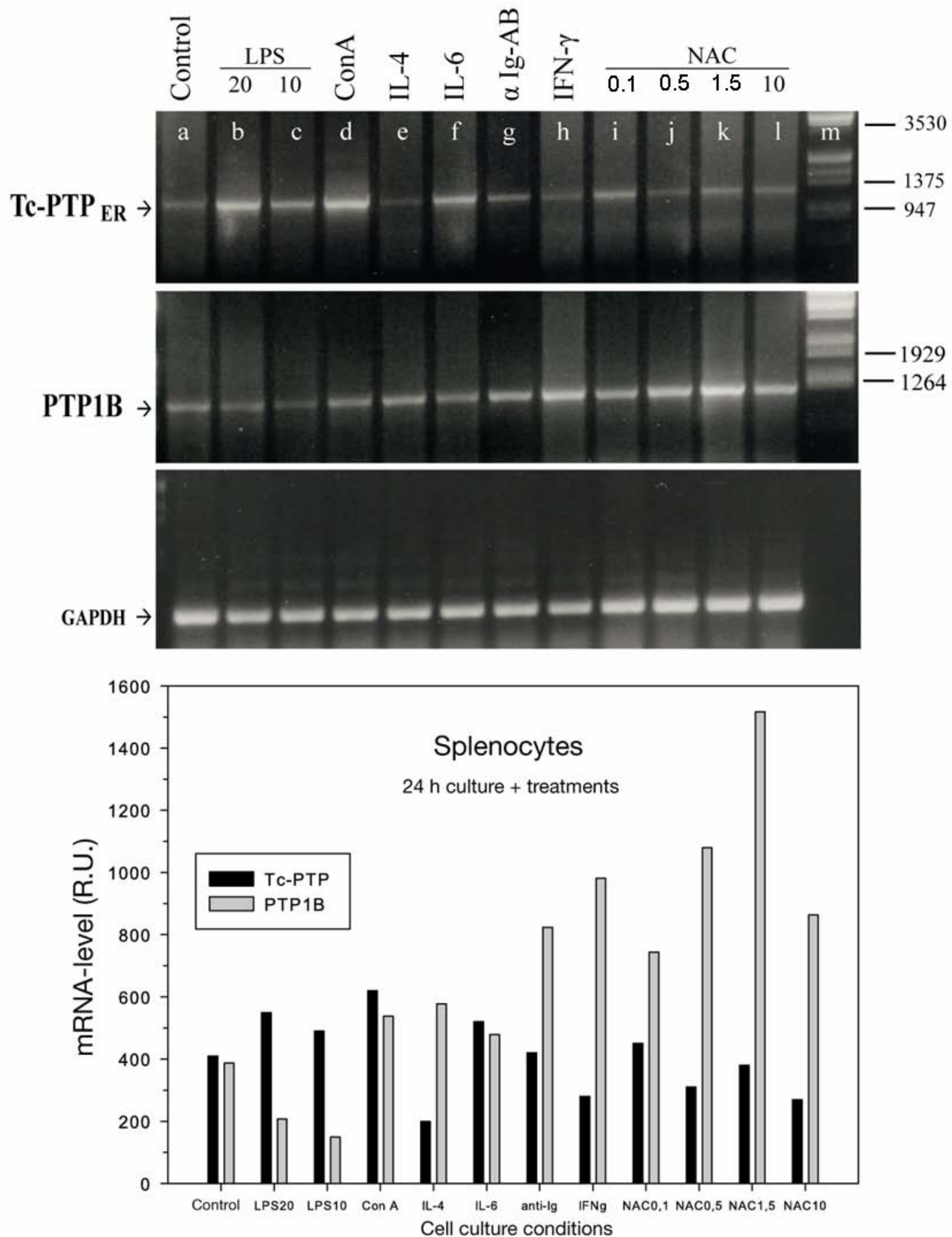

Fig. 5. RT-PCR analysis of the expression of Tc-PTP (ER-form) and PTP1B in cultured pig splenocytes. Splenocytes were cultivated for 24 h (RPMI-10 % FCS), and then for a further 24 h stimulation period: control (unstimulated, lane a); LPS (in μg/ml, lanes b-c); 5 μg/ml ConA (lane d); 100 U/ml IL-4 (lane e); 100 U/ml IL-6 (lane f); 2 μg/ml anti-Ig antibody (lane g); 0.5 μg/ml γIFN (lane h); NAC (mM concentrations, lanes i-l). Primers for Tc-PTP were: Tc-NT-5' + Tc-CT-3'. RT-PCR of GapDH expression is also shown. Densitometric analysis of both PTPs shows the relative amounts of amplified DNA.

The expression of Tc-PTP and PTP1B under both physiological and pathological situations is described thoroughly in the literature [50, 51]. It has been demonstrated that the expression of PTP1B is associated to different metabolic stress situations [52, 53]. Increased expression of PTP1B (or an increased catalytic activity in skeletal muscle, adipose tissue and liver, in humans and animals) has been linked to insulin resistance and obesity. For instance, in experiments with rats, a decrease in PTP1B expression was observed in the liver after food deprivation, whereas the expression of Tc-PTP remained unchanged [28]. Fibroblasts from rats that had been treated with highly concentrated glucose solutions produced a specific increase in PTP1B mRNA. These findings indicate a unique function of PTP1B in the cellular response to metabolic stress [54]. Thus, the control of gene expression of this enzyme would be a physiologically relevant mechanism of regulation in this cellular context. Several investigations showed that the expression levels of Tc-PTP and PTP1B are altered in transformed cells with Bcr-Abl (a fusion Tyr-kinase observed in the early stages of the human chronic myelogenous leukemia, CML) [55]; PTP1B dephosphorylates the PTK Bcr-Abl on Tyr-177. The PTP1B expression increases after a transformation of hematopoietic cells containing the Bcr-Abl fusion protein, which suggests a regulatory role for PTP1B in the control of Tyr phosphorylation(s) at this altered PTK. On the other hand, a down-regulation of the expression of the Tc-PTP has been observed in CML cell lines, which developed a resistance to the inhibitor STI571 [55]. This decrease in the expression of the Tc-PTP culminated in an activatory hyper-phosphorylation of STAT5 [56]. PTP1B showed contradictory results on its gene expression level in various types of cancers. While in esophagus cancer the mRNA level of PTP1B was decreased, in other tumors such as epithelocarcinomas and some gynecological tumors, an over-expression of transcripts of this enzyme was observed [57].

Cytokine receptors are functionally associated with the Jak family of Tyr-kinases, triggering the Jak/STAT signaling [58]. The cytokine-receptor binding ends on the Tyr-phosphorylation and dimerization of different members of the STAT family of transduction factors, which activate gene expression in the immune cells. Over-stimulation of immune cells can lead to malignant proliferation; therefore a 'fine adjustment' of the Tyr-phosphorylations by different members of the PTP family in lymphocytes is expected. The treatment of lymphocytes with H_2O_2 caused similar intracellular effects such as those exerted by extracellular ligands of immune cells. A H_2O_2 exposure of T-lymphocytes produced a fast (seconds after exposure) Tyr-phosphorylation at different intracellular proteins. It has been found that some Tyr-kinases of lymphocytes such as $p56^{lck}$ became autophosphorylated after H_2O_2 exposure and were thus catalytically activated (by phosphorylation of the Tyr-394 in human Lck) [59].

The amount of transcripts of a specific gene may be changed by different mechanisms [60]:

a) modifying the rate of expression of the gene affecting the transcription mechanisms in the nucleus.
b) modification of the rate with which the mature transcripts migrates out from the nucleus.
c) modifying the stability of the mature transcripts in the cell or their cytoplasmic half-life, in dependence with the translation rate, or the degradation of the mature transcripts [61, 62].

There is less information about the cellular control of mRNA stability in members of the PTP family. In the present study, opposed effects were found in the regulation of the expression of Tc-PTP and PTP1B. Such results were particularly pronounced after exposure of cultured splenocytes to the cytokines IL-4 and γIFN. Particular attention should be given to the feasible redox control on the expression of both PTPs in different subsets of splenocytes. H_2O_2 increased markedly the relative amount of Tc-PTP mRNA, while a similar activatory effect was observed for PTP1B after exposure to different concentrations of the antioxidant NAC. Cytokines are synthesized by different cells of the immune system (macrophages, Th1/Th2-lymphocytes) and activate different Jak/STAT signaling pathways [58, 63]. The individual PTP(s) which control the cytokine signaling pathway are still under investigation, as well as the relevance of the expression of different PTPs upon the Jak/STATs pathways in cells of the immune system.

Different redox levels would control the expression of PTPs in lymphocytes; moreover the catalytic PTP activity would also be under intracellular redox

control [64-67]. Some PTPs are selectively inhibited by oxidizing agents (H_2O_2) [68-70]. This could also be of importance at pathological conditions (eg. diabetes) [71, 72]. It has been proposed that the *in vivo* control of PTPs (such PTP1B) includes physiological regulation by H_2O_2 [65, 73, 74]. The production of oxygen-reactive species has long been known as a general defense mechanism of innate immunity. Furthermore, the effect of the redox status upon the transcriptome in different cell types has been described [75, 76]. The extracellular stimulation events that culminate in the generation of intracellular ROS molecules (such as H_2O_2) have been investigated in the last decade [64, 73, 77-79].

The lectin ConA (a classical T lymphocyte mitogen) activates the leukocyte proliferation acting on the intracellular Ca^{2+} levels [80]. Both PTPs augmented their transcript levels after ConA treatment of the splenocytes (probably both PTPs in T lymphocytes), but only Tc-PTP showed an increased expression after LPS treatment of the cells (PTP1B expression was lowered by LPS). LPS is a B cell mitogen, but it also activates T lymphocytes indirectly [81] as well as directly [82]. The biochemical treatments analyzed in this study can act simultaneously on the same cell subtype as well as on different subsets of T or B splenic lymphocytes, generating the observed effects in the expression of both PTPs. Biochemical interactions between activated T-/B-lymphocytes should also be expected in the cultured splenocytes. Further analysis has been performed to investigate the expression of these PTPs in subsets of spleen lymphocytes (Simpfendörfer, in preparation). The cellular distribution of Tc-PTP and PTP1B in splenocytes subsets is also a subject of further analysis in this and other animal models.

H_2O_2 increased the expression of Tc-PTP in splenocytes. The activation of the expression of PTPs by H_2O_2 (Tc-PTP or other) in immune cells has not been reported in the literature. A binding site for the transcription factor NF-κB in the upstream region of the Tc-PTP gene has been described [83]. This transcription factor is activated by H_2O_2 and depends functionally on the intracellular redox status of immune cells [84, 85]. NAC slightly inhibited the Tc-PTP expression; this result could be consistent with a NF-κB control of the transcription of the Tc-PTP, given that this transcription factor can be inhibited by milli-molar NAC concentrations [86].

Tc-PTP should produce an inhibitory effect on the activation of T lymphocytes [16, 87]. Thus, a regulatory role for this enzyme in the proliferation/differentiation of immune cells after mitogenic stimuli could also imply a participation in the apoptotic control of subpopulations of T lymphocytes [88]. Tc-PTP could act as a negative feedback control of proliferation of lymphocytes after mitogenic stimuli [50]. A mutation in the SH2 domain of the transcription factor STAT2 prolonged the Tyr phosphorylation of STAT1 (STAT1 is dephosphorylated by Tc-PTP) [32], an event which promotes IFN-induced apoptosis in hematopoietic cells [88]. Thus, specific Tyr-phosphorylation(s) of different groups of proteins in hematopoietic cells determine the equilibrium between proliferation, differentiation and apoptosis [89, 90]. The enzyme Tc-PTP is related with the cell cycle: the gene transcription of the enzyme is therefore associated with the progress of cell division [91]. In hematopoietic tumors (diffuse large B-cell lymphomas and activated B-cell type tumors), in which IL-4 induced a significant increase in the expression of Tc-PTP, an affected cell cycle regulation has been observed [50]. The ways by which the Tc-PTP may be involved in the proliferation of malignant cells has not been thoroughly investigated (as well as the molecular mechanisms ending in the increased transcription of Tc-PTP) [92]. There is experimental evidence for different tumors (to date by unexplained ways) in which an increased Tc-PTP expression has been associated to cell proliferation [50, 93]. It has been found that an increase in phosphorylation of STATs 1/3 (which are substrates of Tc-PTP, and are deactivated by dephosphorylation by this enzyme), triggered apoptosis (for instance, after the apoptotic induction of HeLa cells by γIFN) [32]. In accordance with such results observed in different cell systems, it can be concluded that a decrease in the Tc-PTP expression triggers apoptosis. In the present study, a decrease in the Tc-PTP expression by γIFN was observed, in agreement with the events triggered by IFN in HeLa cells. Therefore, a possible involvement of a decrease in Tc-PTP expression with apoptosis of immune cells could be proposed. Evidence also indicates an

association of other PTPs (eg. for R-PTPα) with the onset of apoptosis [5, 88].

Further investigation is needed to characterize the functional associations between gene expression of these PTPs with the biochemical/pharmacological control ways of signal transduction in different cell types of the immune system, in particular under different physiological (or pathological) conditions affecting the intracellular redox status, and as potential targets of pharmacological control [23, 94, 95].

CONCLUSION

Mature transcripts of the protein tyrosine phosphatases Tc-PTP and PTP1B were amplified by RT-PCR in swine splenic lymphocytes, which showed marked increases in their expression level after exposure of the cultured cells to increasing concentrations of the mitogen concanavalin A. In the presence of an oxidizing medium, Tc-PTP showed an increased expression (1.5 h after H_2O_2 exposure), whereas PTP1B showed a higher expression after exposure of the cultured splenocytes to the antioxidant N-acetylcysteine for 24 h. This result may indicate an *in vivo* redox control upon the expression of both PTPs during the immune response. Treatments showing markedly opposed results in the expression of both PTPs were LPS (increased expression of Tc-PTP and lower expression of PTP1B), IL-4 and γIFN (marked decrease in the expression of Tc-PTP and increased expression of PTP1B). These results indicate non-redundant roles for both PTPs in the signal transduction events of lymphocyte activation. The regulation of the expression of these non-receptor PTPs could correspond to a control mechanism of lymphocyte apoptosis.

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CONFLICT OF INTEREST STATEMENT

None.

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