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Pentoxifylline inhibits the expression of tissue factor mRNA in endotoxin-activated human monocytes

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Tissue factor (TF) is a transmembrane glycoprotein which, in association with factor VII(a), is the main activator of coagulation. In illnesses such as Gram-negative endotoxemia, circulating monocytes synthesize and express substantial TF activity, resulting in extensive disseminated intravascular coagulation. We investigated the way in which TF is suppressed by pentoxifylline (PTX), and found that PTX down-regulates immunologic TF expression and specific mRNA production in response to LPS. Since TF mRNA stability is not altered, this effect appears to take place at the transcriptional level.

Tissue factor; Monocyte; Pentoxifylline; Endotoxin

1. INTRODUCTION

LPS-activated monocytes synthesize and express on their surface tissue factor (TF), a membrane-bound glycoprotein that serves as the essential cofactor for factor VII(a) in the initiation of coagulation via factors IX and X [1]. Subsequent exposure of blood or plasma to TF-positive monocytes induces pericellular fibrin [2]. The role of TF in triggering endotoxin-induced disseminated intravascular coagulation has been recently emphasized [3,4], and molecules down-regulating its production would be useful therapeutic tools.

Pentoxifylline (PTX) is a methylxanthine derivative [1-(5-oxo-hexyl)-3,7-dimethylxanthine] (Torental, Hoechst) which inhibits phosphodiesterase [5]. Recently, we reported that PTX inhibits the TF procoagulant activity developed by monocytes in vitro in response to endotoxin, apparently through an early increase in intracellular cyclic AMP levels [6].

In this study, we investigated the mechanism regulating the response of TF to PTX in vitro with respect to immunologic TF expression, mRNA levels and mRNA stability.

2. MATERIALS AND METHODS

Peripheral blood monocytes (75–95%) were isolated from the by-products of normal donor plateletpheresis by Ficoll-Hypaque gradient sedimentation and adherence to gelatin/fibronectin-coated Petri dishes [7]. Adherent cells were incubated with 1 μg/ml LPS and with or without PTX, at 37°C under 5% CO_2 for 1–4 h, in RPMI 1640 supplemented as described in [6]. The supernatant was then discarded and cells were washed and stored for less than one week at −80°C until PCA measurement or RNA extraction.

After thawing, the cells were lysed and TF procoagulant activity (PCA) was assayed as described in [6,7]. PCA was expressed as TF milliunits/10^6 monocytes. In some experiments, the percentage of TF-initiated PCA was determined by measuring residual PCA after incubating the cell lysate with 10 μg/ml of a pool of neutralizing TF-monoclonal antibodies (TF8-5G9, TF8-6B4, and TF9-9C3) generously given by T.S. Edgington [8].

Total RNA was extracted by the guanidinium-thiocyanate/cesium chloride method [7]. Filters were hybridized for 24 h at 42°C either subsequently to the labeled TF and β actin cDNA probes or simultaneously with the TF and G3PDH cDNA probes. The autoradiograms were scanned by laser densitometry using the Sebia Preference R apparatus to determine the relative levels of mRNA in treated and untreated monocytes.

cDNA probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by random hexamer primer extension using the Amersham Multiprime kit. The 641 bp TF cDNA probe kindly provided by T.S. Edgington [9] was excised from plasmid pUC8 with *Eco*RI. The 822 bp β actin cDNA probe was excised from plasmid pGEM3 with *Eco*RI and *Hin*dIII; the latter is a murine probe which cross-reacts with human actin mRNA and was kindly provided by Dr. Alvarado (Institut Pasteur, Paris). The G3PDH probe was purchased from Clontech (Palo Alto, CA).

The binding of an anti-human TF murine monoclonal (IgG) antibody (MoAb4, Corvas, San Diego, CA) was assessed by flow cytometric analysis [7]. Monocytes were gated using anti-CD14-positive cells detected after incubation with a phycoerythrin-conjugated anti-CD14 mouse monoclonal antibody (Immunotech, Luminy, France) [7].

3. RESULTS AND DISCUSSION

We confirmed here that PTX inhibits the generation of TF-procoagulant activity by LPS-exposed monocytes, in a concentration-dependent manner, (Fig. 1). The PCA was identified as TF since more than 99% was

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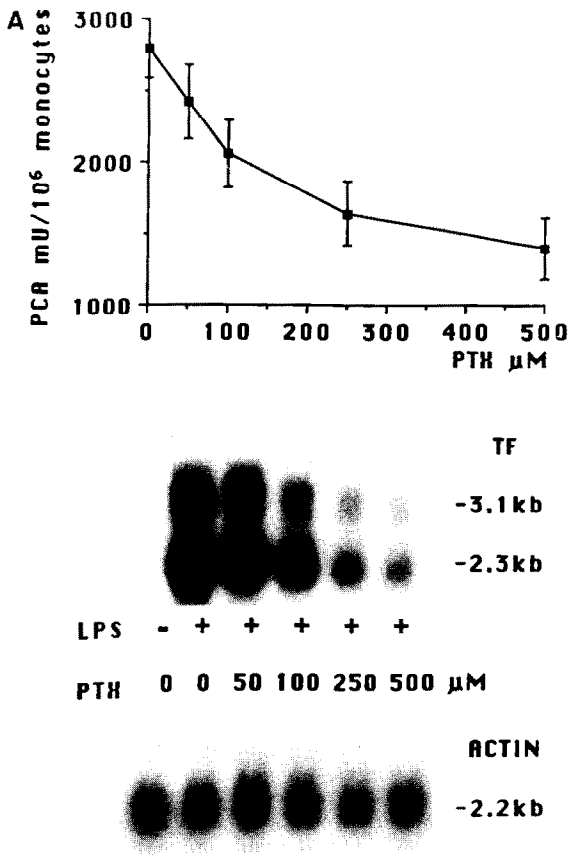

Fig. 1. PTX induced parallel, concentration-dependent inhibitions of LPS-induced PCA (A) and TF mRNA (B). Five μg of total RNA was analysed by Northern blotting. RNA was extracted from untreated monocytes, or from cells exposed for 4 h to 1 μg/ml LPS or to LPS + PTX at 50 to 500 μM. The membranes were hybridized to TF and actin probes. Monocyte PCA was determined in the same system (A); (mean ± S.D.; n = 3).

inhibited by a cocktail of monoclonal antibodies to human TF (data not shown).

Quantitation by flow cytometric analysis with a specific monoclonal antibody showed that TF protein expression on the cell surface and TF functional activity was decreased by PTX treatment (Fig. 2). The median fluorescence level was 30 in the absence of stimulation, 200 after LPS treatment and 69 in the presence of PTX at 500 μM (65% decrease). PCA measured in the same experiment decreased from 6,346 to 2,346 mU/10^6 monocytes (63% decrease). This is notable since the existence of TF pathway inhibitor (TFPI) mRNA [10] in both resting and LPS-stimulated monocytes suggests that this inhibitor may play a role in the decrease in TF activity. In addition, TF activity may be influenced by membrane composition, including increased phosphatidylserine content in the outer leaflet of the plasma membrane [11].

TF mRNA was not detectable in unstimulated monocytes (Fig. 1). In contrast, exposure to 1 μg/ml LPS for 4 h was associated with high TF mRNA levels. The addition of 50–500 μM PTX to monocytes during LPS stimulation was associated with a concentration-dependent decrease in TF mRNA (Fig. 1). This involved both the 3.1- and 2.3-kilobase species. No changes in β actin mRNA content were observed in control or drug-treated cells. Similarly, TF mRNA levels in monocytes exposed to 1 μg/ml LPS for 0–4 h were compared with and without PTX (500 μM) pretreatment (Fig. 3). Upon LPS stimulation, TF mRNA increased progressively from 0 to 4 h (Fig. 3), as previously reported by Gregory et al. [12]. When PTX was added, TF mRNA was un-

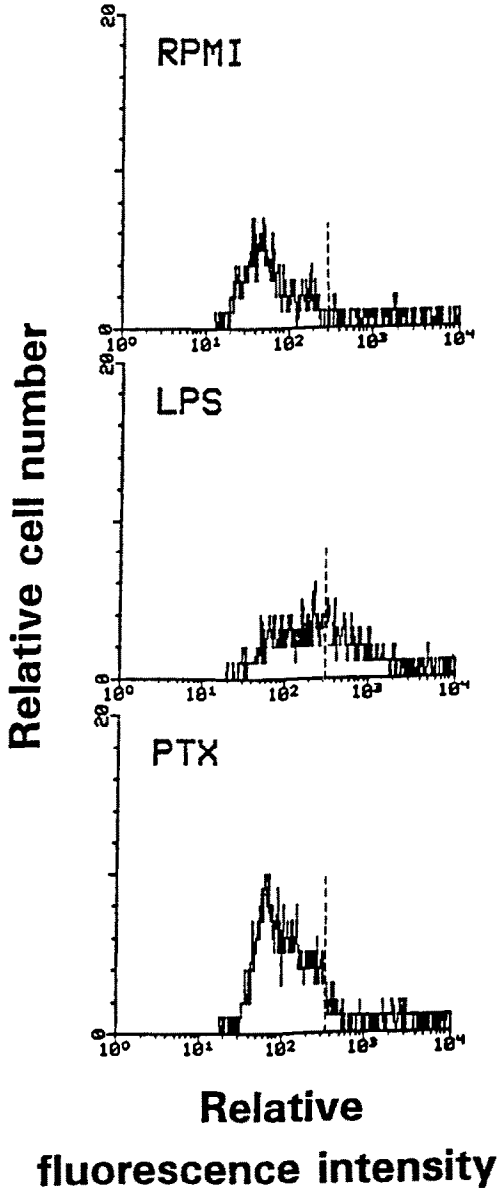

Fig. 2. Down-regulation of LPS-induced TF antigen expression analyzed by flow cytometry in monocytes treated with PTX. Monocytes were incubated in culture medium (RPMI) or treated with LPS (1 μg/ml) alone or LPS + 500 μM PTX (PTX). The dotted line shows the maximal fluorescence intensity obtained with cells incubated in culture medium alone.

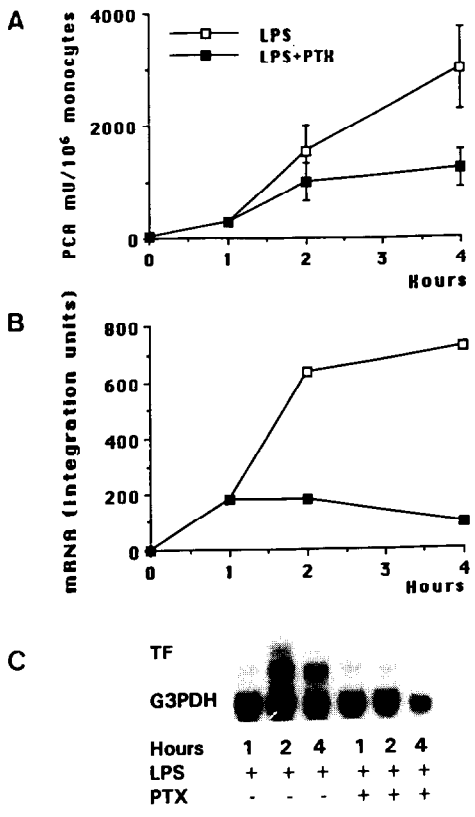

Fig. 3. Kinetics of PTX inhibition of LPS-induced PCA (A) and TF mRNA (C) from 0 to 4 h. RNA was extracted from freshly isolated monocytes (not shown) after 1, 2 and 4 h incubation with 1 μg/ml LPS alone or LPS + 500 μM PTX. Autoradiograms were scanned by laser densitometry and values were calculated relative to the G3PDH control (B). The kinetics of monocyte PCA was determined in the same system (A); (mean± S.D.; n = 3).

modified at the first hour but was profoundly decreased at 2 and 4 h. The PCA of similarly treated monocytes was determined concomitantly. PCA increased progressively from 0 to 4 h after LPS stimulation alone (Fig. 3); after the addition of PTX, it fell by about 50% at 2 and 4 h. The kinetics of mRNA inhibition by PTX was similar to that observed for PCA inhibition.

The rate of mRNA degradation is an important potential regulator of gene expression. The half-life of the TF mRNA, with or without PTX pretreatment, was about 3.25 h. The yield of total RNA decreased similarly in both conditions (Fig. 4). This indicates that PTX does not alter the half-life of TF mRNA and strongly suggests an effect of PTX at the transcriptional level.

PTX decreases tumor necrosis factor α mRNA accumulation in LPS-activated monocytes [13] and prevents thrombomodulin mRNA suppression in tumor necrosis factor α-exposed endothelial cells [14]. In both cases the effect of PTX may occur partly via an increase in intracellular cAMP levels. On the basis of our previous results [6], a similar mechanism is likely to be involved in the inhibition of LPS-induced generation of TF by PTX. Here, we show that the functional and immunological alterations of LPS-induced TF, as well as the TF mRNA suppression induced by PTX, present similar characteristics to those reported to be induced by pharmacological agents that elevate intracellular cAMP levels [7].

Despite the strong evidence favoring a cAMP-dependent mechanism of PTX action, other possibilities should also be considered. PTX induces structural defects in the neutrophil cytoskeleton [15] and we have reported the inhibitory effect of antimicrotubular agents on monocyte TF production [16]. Recently, PTX was reported to be a scavenger of hydroxyl radicals and to have protective effects against hydroxyl radical-induced damage [17]. Since the effect of such radicals has been suggested to be involved in the LPS-induced transactivation of nuclear factor-kB [18], this mechanism might also contribute to TF mRNA down-regulation by PTX.

Since TF probably has a prominent role in the initiation of coagulation [19], the suppression of TF production by PTX likely contributes strongly to its antithrombotic effect [20]. Indeed, PTX is beneficial in septic shock in several animal experimental models [21,22]. Fletcher et al., however, insisted on the fact that PTX treatment can improve or worsen survival from LPS shock in rats, depending on the dose and dosing schedule [23].

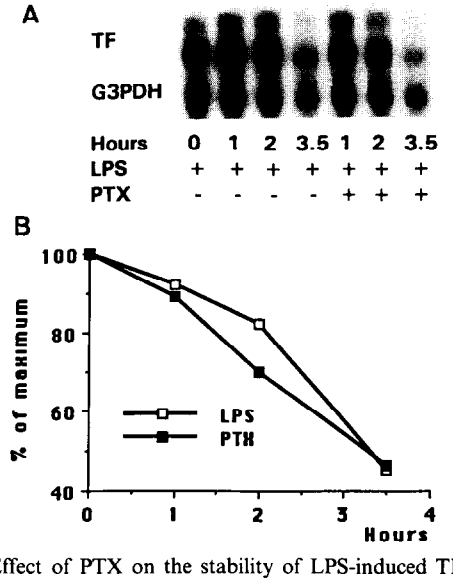

Fig. 4. Effect of PTX on the stability of LPS-induced TF mRNA. Monocytes were exposed to LPS for 2 h to induce TF mRNA. The addition of actinomycin D (10 μg/ml) was followed by the addition of 100 μM PTX. Total RNA was harvested at the indicated times after the addition of actinomycin D and analyzed by Northern blotting (A). Autoradiograms were scanned by laser densitometry. The stability of the 2.3 kb TF mRNA species is shown (B): □ LPS alone, ■ LPS + PTX.

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