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Pentoxifylline Inhibition of Procoagulant Activity Generated by Activated Mononuclear Phagocytes

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SUMMARY

When appropriately stimulated, monocytes are able to initiate blood coagulation through the membrane expression of tissue factor. This procoagulant activity is thought to play a role in activating coagulation in response to inflammatory stimuli *in vivo*. We found that pentoxifylline, a methylxanthine derivative already reported to regulate some monocyte functions, inhibits the procoagulant activity developed by U937 cells and monocytes *in vitro* in response to endotoxin. This effect was accompanied by an early increase in intracellular levels of cyclic AMP and was mimicked by compounds that induce an increase in the level of cyclic AMP levels. These results suggest that the suppressive effect of pentoxifylline occurs at least in part via an increase in intracellular cyclic AMP levels.

Recent work has shown that, on exposure to a variety of stimuli (bacterial endotoxin, immune complexes, proteolytic complement fragments, cytokines, etc.), blood monocytes and tissue macrophages produce and express on their surface a PCA (1, 2). This PCA has usually been identified as TF, a membrane protein that is an essential cofactor in triggering the coagulation of blood via the extrinsic pathway. The induction of pericellular fibrin by specifically activated monocytes has also been reported (3, 4). In view of this ability to produce PCA and induce fibrin thrombi, mononuclear phagocytes have been implicated in the activation of intravascular and extravascular coagulation occurring in malignancy, endotoxemia, and immunological diseases (5–7).

PTX is a methylxanthine derivative [1-(5-oxo-hexyl)-3,7-dimethylxanthine] (Torental; Hoechst), which has already been shown to modulate one monocyte function, LPS-induced tumor necrosis factor production (8). Moreover, PTX, alone or in combination with low doses of acetylsalicylic acid, inhibits experimental thrombus formation *in vivo* (9). For these reasons, it was of interest to study the effect of PTX on endotoxin-induced TF generation. Human blood monocytes and the U937 monocyte-like cell line established from the pleural effusion of a patient with histiocytic lymphoma (10) were used for this study. These latter cells have many properties in common with normal monocytes (10, 11), including the expression of PCA following LPS stimulation (12). The mechanisms of the observed effects were investigated.

Materials and Methods

Isolation of blood mononuclear cells. Blood was collected from healthy volunteers into preservative-free lithium heparinate (10 IU/ml of blood). Mononuclear cells were isolated by the Ficoll-Hypaque gradient technique, as modified by Ollivier *et al.* (13). In order to minimize platelet contamination of the mononuclear suspension, cells were washed with 5 mM phosphate-buffered saline-EDTA, as described by Altieri *et al.* (14), and finally suspended in RPMI 1640 medium (GIBCO) supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate, and 10% heat-inactivated fetal calf serum (GIBCO). This procedure gave more than 95% mononuclear cells, including 20–30% monocytes, 60–70% lymphocytes, and less than 10 platelets/monocyte. Monocytes were identified by cytochemical reactivity for α-naphthyl acetate esterase, as described by Tucker *et al.* (15).

U937 cells. U937 cells (obtained from G. Lemaire, Université d'Orsay, France) were grown in RPMI 1640 supplemented as for mononuclear cells, in a 5% CO_2 humidified atmosphere at 37°. Cells were initially cultured at 1×10^5/ml and were passaged every 2–3 days. Cell viability was determined using trypan blue exclusion.

Induction of procoagulant activity. Mononuclear cell suspensions (containing 10^6 monocytes/ml) or U937 cells (2×10^6/ml) were incubated at 37° under 5% CO_2 in sterile culture tubes, for 14–16 hr, with or without endotoxin LPS from *Escherichia coli* (055-B5; Sigma), dbcAMP (Sigma), IBMX (Sigma), or PTX. The total volume of the incubation medium was 500 μl. Cells were washed and stored for less than 1 week at −80° until PCA measurement. It was verified that PCA activity was not altered under these storage conditions.

PCA assay. Immediately before PCA was assayed, the cells were lysed with 16 mM octyl-β-D-glycopyranoside for 10 min at 37°, as described by Tsao *et al.* (16).

PCA was evaluated using a one-step plasma recalcification time, measured as follows: 100 μl of 25 mM $CaCl_2$ were added to 100 μl of a pool of citrated normal human platelet-poor plasma mixed with 100 μl of the test suspension, and the clotting time was recorded. Clotting

ABBREVIATIONS: PCA, procoagulant activity; dbcAMP, 2′-*O*-dibutyryl adenosine 3′5′-cyclic monophosphate; IBMX, isobutylmethylxanthine; LPS, lipopolysaccharide; PTX, pentoxifylline; TF, tissue factor.

times were compared with those of serial dilutions of a standard TF preparation (Thrombomat; Biomérieux). A sample of this preparation containing 1000 milliunits/ml TF caused normal plasma to clot in 50 sec. PCA was expressed as TF milliunits/10^6 monocytes or milliunits/2×10^6 U937 cells.

cAMP assay. cAMP was measured in U937 cells at 1, 5, 20, and 30 min after the addition of LPS, PTX, or a combination of these agents, using a radioimmunoassay kit (Rianen cAMP [I^{125}]).

Cell viability. Cell viability was determined in terms of trypan blue exclusion and by measurement of lactate dehydrogenase activity in the cell-free supernatants and in the cell lysates, according to the method of Beutler (17).

Statistical analysis. Each experiment was performed in duplicate, using monocytes from various donors. Results are given as mean ± standard error of n separate experiments. Data were compared using Wilcoxon's paired t test.

Results

Effect of PTX on the PCA of human blood monocytes. Freshly collected mononuclear cells had no detectable PCA (clotting times, >160 sec); after incubation, the PCA levels of untreated cells and of LPS-treated cells were 104 ± 61 and 1992 ± 257 milliunits/10^6 monocytes, respectively (mean ± SE of six separate experiments). PTX inhibited the generation of PCA in a concentration-dependent manner. A negative correlation ($p < 0.01$) was found between PTX concentrations and monocyte PCA (Fig. 1). This inhibitory effect was statistically significant ($p < 0.01$) with PTX concentrations of 10 μM and above. PTX at a concentration of 500 μM inhibited 50% of total PCA measured following cell disruption. At these PTX concentrations, more than 95% of the cells excluded trypan blue at the end of the stimulation period. The release of the cytosol marker lactate dehydrogenase into the supernatant of PTX- and LPS-treated cells was similar to that of untreated cells and was already below 10%.

Effects of PTX on the PCA of U937 cells. The PCA of unstimulated U937 cells was 327 ± 14 milliunits/2×10^6 cells; the PCA of cells treated with 1 μg/ml LPS was 463 ± 15 milliunits/2×10^6 cells. PTX significantly ($p < 0.05$) inhibited the LPS-induced generation of PCA to 449 ± 16, 406 ± 17, and 362 ± 8 milliunits at concentrations of 100, 250, and 500 μM, respectively. A negative correlation ($p < 0.01$) was found between PTX concentration and cell PCA (Fig. 2).

cAMP levels. cAMP levels were determined in U937 cells at 1, 5, 20, and 30 min after the addition of PTX (500 μM) and/or LPS (10 μg/ml). A mixture of IBMX and isoproterenol was used as positive control treatment. As shown in Fig. 3, cAMP levels were not modified by the addition of LPS. In contrast, PTX alone or in association with LPS led to a clear cut increase in cAMP levels, with a peak at 1 min and subsequent decreases at 5, 20, and 30 min.

As shown in Fig. 4, increasing concentrations of PTX (from 50 to 500 μM) led to the generation of increasing concentrations of intracellular cAMP at 1 min. PTX concentrations and cAMP levels were positively correlated ($p < 0.001$) in the range of 50 to 500 μM.

Effect of IBMX and dbcAMP on LPS-stimulated monocyte PCA. In order to assess whether the inhibition of PCA generation could be related to the observed increase in cAMP levels, the effects of dbcAMP (a cAMP analogue) and IBMX (which promotes the accumulation of intracellular cAMP) were investigated. As shown in Fig. 5, 500 μM dbcAMP and IBMX significantly ($p < 0.01$) reduced the ability of LPS to induce

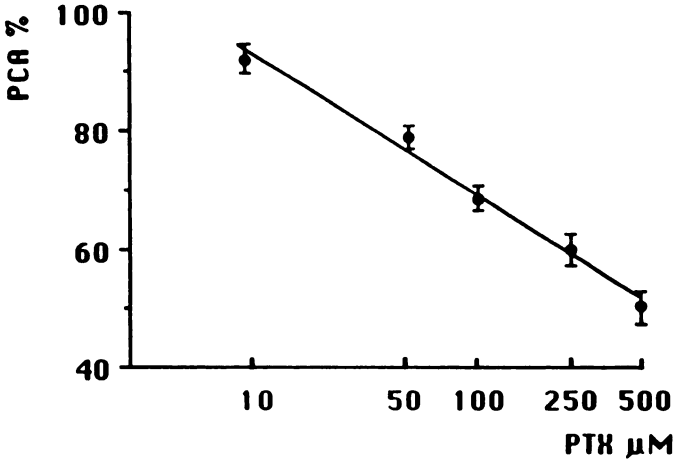

Fig. 1. Negative correlation between the logarithm of PTX concentrations and the PCA induced by 0.1 μg/ml LPS in a suspension of mononuclear cells ($r = -0.98$, $p < 0.001$, slope = −24.5). PCA is expressed in percentage of the PCA induced by LPS without PTX (1992 ± 257 milliunits/10^6 monocytes). Each *point* represents the mean of six separate experiments performed in duplicate.

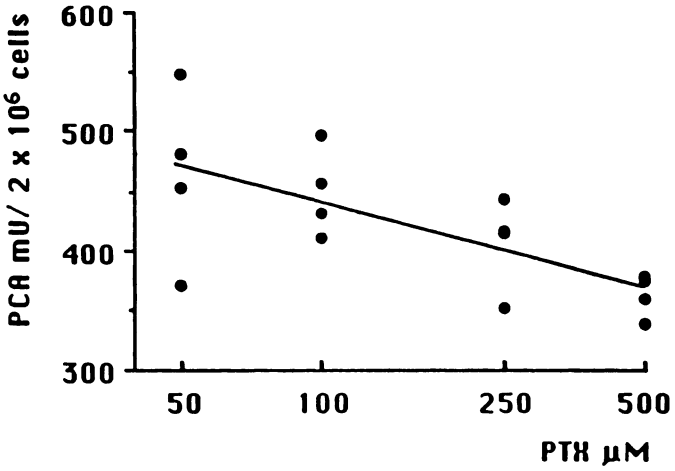

Fig. 2. Negative correlation between the logarithm of PTX concentrations and LPS-induced PCA in a suspension of U937 cells ($r = -0.69$, $p < 0.01$, slope = −101.5).

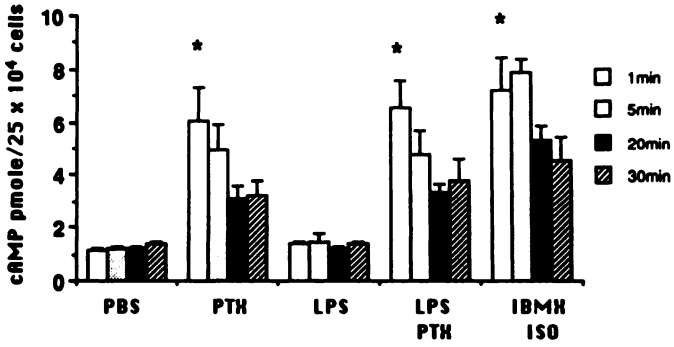

Fig. 3. cAMP levels in U937 cells 1, 5, 20, and 30 min after the addition of phosphate-buffered saline (*PBS*), LPS (10 μg/ml), PTX (500 μM), LPS plus PTX, or IBMX plus isoproterenol (*ISO*) (each *point* represents the mean ± standard error of three separate experiments performed in duplicate). *, PTX: $p < 0.01$ versus phosphate-buffered saline; *, LPS-PTX: $p < 0.01$ versus LPS; *, IBMX-isoproterenol: $p < 0.01$ versus phosphate-buffered saline.

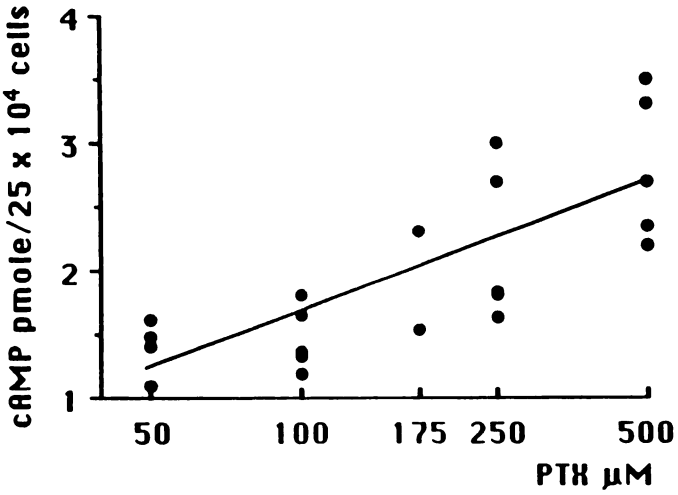

Fig. 4. Correlation between the logarithm of PTX concentrations and intracellular cAMP levels generated at 1 min in U937 cells ($r = 0.78$, $p < 0.001$, slope = 1.45). Each *point* represents the mean result of one experiment performed in duplicate.

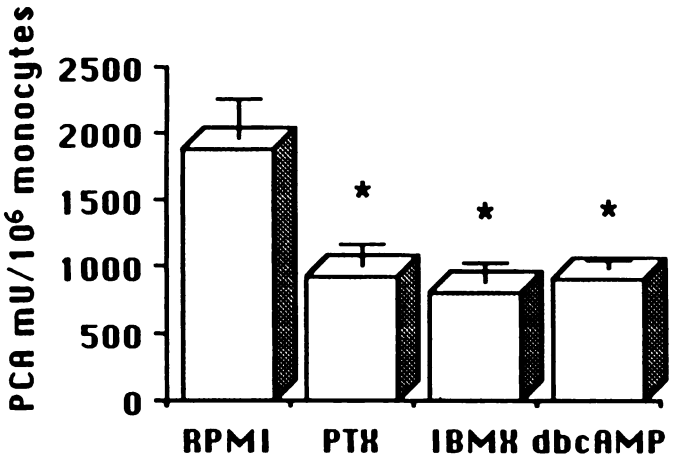

Fig. 5. Effects of 500 µM PTX, IBMX, and dbcAMP on the PCA of LPS-stimulated monocytes (mean ± standard error of four separate experiments). *, p 0.01 versus untreated cells (RPMI).

monocyte PCA. This inhibitory effect was of the same order of magnitude as that induced by 500 µM PTX.

Moreover, increasing concentrations of dbcAMP (from 10 to 500 µM) induced a concentration-dependent inhibition of monocyte PCA generation; as shown in Fig. 6, the two parameters were negatively correlated ($p < 0.001$).

Discussion

In response to various stimuli, including bacterial endotoxin, cytokines, immune complexes, and complement components, cells of the mononuclear phagocyte system synthesize and express TF on their membrane, resulting in the activation of intra- or extravascular coagulation.

PTX, a methylxanthine derivative, was initially found to abrogate claudication in patients with peripheral vascular diseases (18). More recently, it has been shown to inhibit experimental thrombus formation *in vivo* (9).

In this study, we found that PTX caused a concentration-dependent decrease in endotoxin-induced PCA of human blood monocytes at concentrations in the range of 10 to 500 µM. A strong negative correlation ($p < 0.01$) was found between PTX

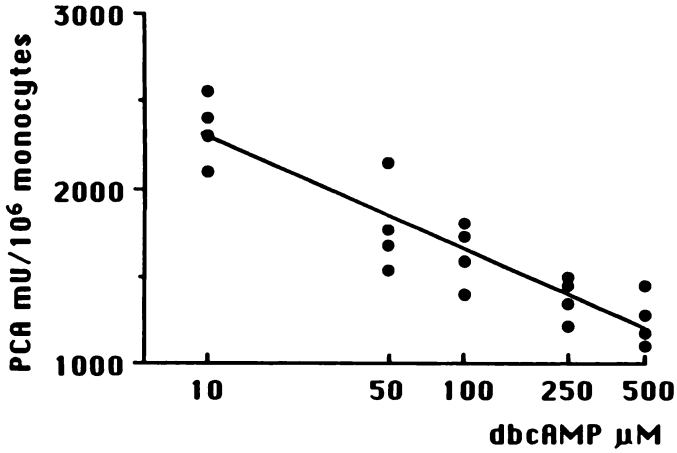

Fig. 6. Negative correlation between the logarithm of dbcAMP concentrations and LPS-induced monocytes PCA ($r = -0.91$, $p < 0.001$, slope = −642.3).

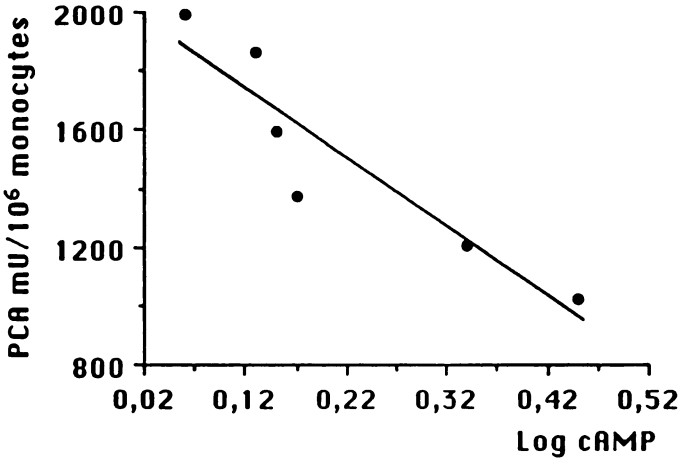

Fig. 7. Negative correlation between LPS-induced monocyte PCA and the logarithm of corresponding cAMP values in the presence of increasing concentrations of PTX (0, 10, 50, 100, 250, and 500 µM, from *left* to *right*) ($p < 0.01$, $r = -0.86$, slope = −2377).

concentration and monocyte PCA. This inhibitory effect was also observed in the range of 100 to 500 µM when U937 cells were used. These results are in accordance with those of Lyberg (19), who found that IBMX, another methylxanthine, inhibited the PCA induced by various stimuli. Interestingly, Beretz *et al.* (20) recently reported that PTX (1–10 mM) inhibited the increase in PCA caused by tumor necrosis factor in endothelial cells. This property, evidenced in both monocytes and endothelial cells, could play a role in the antithrombotic action of the drug. Other mechanisms may contribute to this effect, because PTX has been shown to act at several levels of the hemostatic system, inhibiting platelet function (21, 22) and increasing prostacyclin production by endothelial cells (23).

PTX plasma concentrations during intravenous infusion of the drug reach about 10 µM,[1] i.e., equivalent to the lowest concentration of the drug inducing a small but significant inhibition of monocyte PCA in our experiments. Moreover, it has been reported in platelet aggregation studies that alteration of cell functions may be observed *in vivo* at PTX concentrations below those that were effective *in vitro* (21). The prolonged

[1] C. Gibert, Service de Réanimation, Hôpital Bichat, Paris, personal communication.

presence of a low PTX concentration might be as effective as a higher concentration for a short period. PTX might, thus, prove useful in clinical situations such as gram-negative sepsis, malignancy, and systemic lupus erythematosus (5, 7, 24), where increased monocyte PCA is involved.

PTX is a methylxanthine that acts mainly through an increase in intracellular cAMP levels but also via other mechanisms such as actin depolymerization, leading to a decrease in intracellular actin content (25). Because PTX also induced a decrease in endotoxin-induced PCA of U937 cells, this line was used to measure intracellular cAMP levels in the presence of PTX. Our results confirmed the occurrence of an increase in cAMP levels (26) peaking rapidly (1 min) after the addition of PTX to the culture medium, with subsequent decreases at 5, 20, and 30 min. In order to assess whether the inhibition of PCA generation could be related to the observed increase in cAMP levels, the effects of IBMX (a phosphodiesterase inhibitor) and of dbcAMP (a cell-penetrating cAMP analogue) were investigated. Our results show that both drugs, which, by different mechanisms, induce an increase in intracellular cAMP, reduced LPS-stimulated PCA. The production of interleukin-6, interleukin-1α, interleukin-1β, and tumor necrosis factor-α has been reported to be selectively regulated in monocytes by agents that increase intracellular cAMP levels (27). cAMP would act by selectively regulating the expression of the genes encoding inflammatory monokines (28). Our results suggest that cAMP may also be involved in the mechanism of PTX-induced inhibition of LPS-induced monocyte PCA. Indeed, we observed highly significant correlations between (a) PTX concentrations and the inhibition of monocyte PCA ($p < 0.001$), (b) PTX concentrations and intracellular cAMP levels ($p < 0.001$), and (c) dbcAMP concentrations and the inhibition of monocyte PCA ($p < 0.001$). Finally, LPS-induced monocyte PCA in the presence of increasing PTX concentrations correlated strongly ($p < 0.001$) with corresponding cAMP values (Fig. 7). These correlations corroborate the link between increasing cAMP and PCA inhibition. However, because PTX substantially increases cAMP but only partially inhibits LPS-induced PCA, we cannot exclude the involvement of another cAMP-independent pathway in PCA inhibition.

Methylxanthines are known to act not only by inhibiting cytoplasmic phosphodiesterase but also, at the plasma membrane level, by inhibiting adenosine receptors in a competitive manner (29). The stimulation by adenosine of specific adenosine receptors either inhibits (A1 receptors) or stimulates (A2 receptors) adenylate cyclase activity (29). Theophylline has been reported to block completely the inhibitory effect of adenosine analogues on macrophage PCA, whereas theophylline alone has no effect (30). The PTX-induced increase in cAMP levels might be secondary not only to inhibition of phosphodiesterase but also to its binding to adenosine receptors. In this respect, the study of the binding characteristics of PTX to monocytes/macrophages might be extremely interesting, together with the relationship between these characteristics and PTX effects on monocyte PCA.

In conclusion, PTX inhibits the generation of endotoxin-induced PCA. Furthermore, our results suggest that this effect is mediated via an increase in intracellular cAMP. These findings may have important implications in the clinical context, where the triggering of monocyte or macrophage PCA can lead to deleterious effects. Nevertheless, it remains to be shown *in vivo* that PTX can inhibit PCA induction.

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