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Tissue plasminogen activator inhibits P-glycoprotein activity in brain endothelial cells

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Abstract

Tissue plasminogen activator (0.01–30 μg/ml) dose-dependently inhibited the functional activity of P-glycoprotein, assessed by rhodamine 123 accumulation in GP8 immortalized rat brain endothelial cells, but this effect was unrelated to its proteolytic activity. Elevation of intra-endothelial cyclic AMP concentration and stimulation of protein kinase C increased P-glycoprotein activity in GP8 cells and also attenuated the tissue plasminogen activator-induced inhibition. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Blood–brain barrier; P-glycoprotein; Tissue plasminogen activator

Tissue plasminogen activator (tPA), a glycoprotein with serine protease activity, dissolves clots by converting plasminogen into active plasmin. Intravenous administration of tPA improves the clinical outcome of patients with acute ischaemic stroke, but this treatment is also associated with a relatively high incidence of intracranial haemorrhage (Del Zoppo et al., 1998). The question whether extravascular tPA initiates neuronal cell death by promoting excitotoxicity (Wang et al., 1998), or whether it protects neurons against toxic insult (Kim et al., 1999), is still under debate. Non-proteolytic modes of actions might also be involved in the effects of tPA in the central nervous system (Kim et al., 1999). Although the release of plasminogen activators and their natural inhibitors is tightly regulated in cerebral microvessels, the effect of exogenous tPA on the functional and morphological integrity of the blood–brain barrier is not yet known (Del Zoppo et al., 1998). P-glycoprotein, a 170-kDa protein with ATP-dependent pump activity, is one of the major efflux systems of the luminal membrane of the blood–brain barrier responsible for protecting the brain from drugs and xenobiotics (Tsuji and Tamai, 1997). In the present study, the effect of tPA on the functional activity of P-glycoprotein was investigated in cerebral endothelial cells.

Cells of the GP8 rat brain endothelial cell line immortalized by SV40 large T (Greenwood et al., 1996) were cultured in 24-well plates and used between passages 15–22, as has been described in detail (Deli et al., 2000). GP8 cells show both general endothelial and specific blood–brain barrier characteristics, including the expression of P-glycoprotein (Greenwood et al., 1996; Regina et al., 1999). The functional activity of P-glycoprotein was determined by measuring the cellular accumulation of rhodamine 123 (all reagents from Sigma, unless otherwise specified) according to the method of Fontaine et al. (1996). In brief, endothelial monolayers were incubated with 0.5 ml Dulbecco's phosphate-buffered saline supplemented with 10 mM HEPES containing rhodamine 123 (5–20 μM) for 15–120 min. Then, the solution was removed and the endothelial cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 0.2 N NaOH (0.5 ml). The rhodamine 123 content was determined by fluorescence spectrophotometry (excitation:

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505 nm, emission: 534 nm, slit widths: 1.5 nm) using a Shimadzu RF500 spectrofluorophotometer. P-glycoprotein activity was also determined in GP8 monolayers receiving dexamethasone (1 μM), a regulator of P-glycoprotein through protein kinase C activation (Regina et al., 1999), and in primary rat brain endothelial cells isolated and cultured as described earlier (Deli et al., 2000). To assess functional P-glycoprotein activity, cyclosporin A (2–10 μM) and verapamil (100 μM) were added to monolayers. We used a recombinant tPA analogue (0.01–30 μg/ml), in which amino acids 92–173 were deleted and which contained a point mutation $Arg^{275} \rightarrow Glu$ (Yamanouchi Pharmaceuticals), but the results were confirmed with single-chain tPA (Sigma). Drugs used alone, or in combination with tPA (10 μg/ml), were plasminogen activator inhibitor-1 hexapeptide (10 μM; Peninsula Laboratories), serine protease antagonist 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; 200 μM), epidermal growth factor (1 μg/ml), 8-(4-chlorophenylthio)-cyclic AMP (250 μM) in combination with phosphodiesterase inhibitor 4-(3-butoxy-4-methoxy-benzyl)imidazolidin-2-one (RO 20-1724; 17.5 μM, Calbiochem), specific protein kinase A inhibitor *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89; 5 μM, Calbiochem), protein kinase C stimulator 12-*O*-tetradecanoylphorbol 13-acetate (160 nM) and inhibitor 3-[1-(dimethyl-aminopropyl)indol-3-yl]-4-(indol-3-yl)maleimide (bisindolylmaleimide I; 0.1 μM), as well as L-arginine (2 mM), a component of most tPA products. Each result presented is as the mean ± S.D. Statistical analysis was performed using analysis of variance followed by two-sided Dunnett's *t* test.

Our data indicates that GP8 brain endothelial cells have a functional P-glycoprotein system, and that typical efflux inhibitors can increase the intra-endothelial accumulation of rhodamine 123, a P-glycoprotein ligand (Fig. 1). P-glycoprotein activity was about 40% less in GP8 cells than in primary brain endothelial cells (data not shown). Though tPA dose-dependently inhibited the efflux of rhodamine 123 from GP8 cells, natural and synthetic inhibitors of proteolysis did not prevent this effect. Our results do not support the involvement of epidermal growth factor-like activity or L-arginine (Fig. 1). Elevation of intra-endothelial cyclic AMP concentration and stimulation of protein kinase C increased the P-glycoprotein activity and these treatments also attenuated the tPA-induced changes, whereas protein kinase A inhibition was without effect (Fig. 1). We obtained similar results with these drugs on GP8 cells untreated with dexamethasone (data not shown).

This report is the first to describe that tPA, a glycoprotein with multiple pharmacological actions, inhibits P-glycoprotein at the blood–brain barrier. ATP depletion caused by cerebral ischemia decreases P-glycoprotein activity, but the activity recovers within 30 min of reperfusion (Tsuji and Tamai, 1997). If tPA, which is used for fibrinolysis in ischaemic stroke, could prolong P-glycoprotein inhibition, it would enhance the penetration of xenobiotics into the brain. If tPA induces a similar inhibition in other cells with a high P-glycoprotein activity, then tPA may have pharmacological potential in tumour therapy, too.

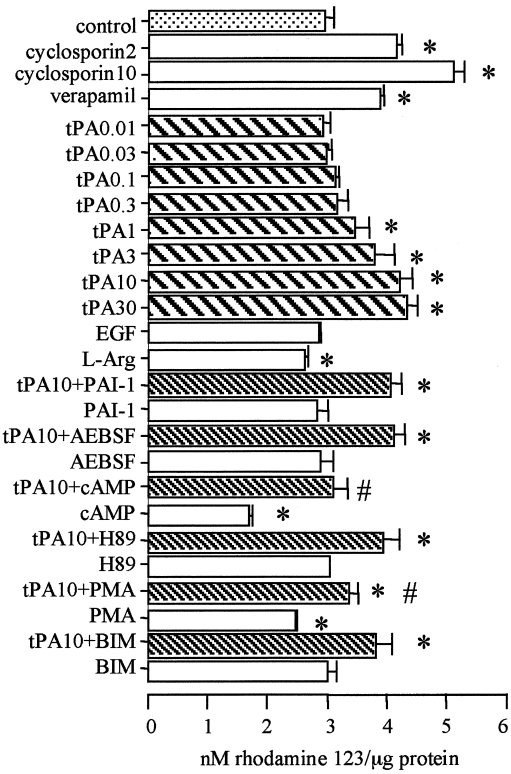

Fig. 1. Effects of tissue plasminogen activator (tPA; 0.01–30 μg/ml) on the P-glycoprotein activity induced by dexamethasone (1 μM) in GP8 cells. Cellular rhodamine 123 content, measured after 120 min of incubation, was expressed as ng rhodamine 123/μg protein. Drug treatments were cyclosporin A (2–10 μM), verapamil (100 μM), epidermal growth factor (EGF, 1 μg/ml) , L-arginine (2 mM), plasminogen activator inhibitor-1 (PAI-1; 10 μM), 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; 200 μM), and agents to increase the intra-endothelial cyclic AMP (cAMP) level, namely 8-(4-chlorophenylthio)-cyclic AMP (250 μM) plus RO 20-1724 (17.5 μM), H-89 (5 μM), 12-*O*-tetradecanoylphorbol 13-acetate (PMA; 160 nM), and bisindolylmaleimide I (BIM; 0.1 μM). Each result presented is the mean ± S.D. Statistical analysis was performed using analysis of variance followed by two-sided Dunnett's *t* test, $^{*}P < 0.05$ compared to control monolayers, $\#P < 0.05$ compared to monolayers treated with 10 μg/ml tPA alone.

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