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INHIBITION OF THE THROMBOPLASTIN RESPONSE OF ENDOTHELIAL CELLS *IN VITRO*

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Abstract—Confluent monolayers of human umbilical vein endothelial cells in culture responded with a 5-fold increase in thromboplastin (tissue factor) synthesis when exposed to 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (50 ng/ml) or endotoxin (ETX) (25 μg/ml) for 16 hr. This induced thromboplastin synthesis was markedly inhibited by exposure of the cells to two different phosphodiesterase inhibitors, methylisobutylxanthine (MIX) and rac-4(3-butoxy-4-methoxybenzyl)-2-imidazole idinone (RO-20-1724) and to the transmethylation inhibitors 3-deazaadenosine (DZA) and 1-homocysteine thiolactone (Hcy) in combination. It was slightly (TPA) or not at all (ETX) inhibited upon exposure of the cells to the intracellular calcium antagonist 8-(*N*,*N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8). However, in the presence of MIX TMB-8 had a moderate additional inhibitory effect on TPA-induced thromboplastin response.

The thromboplastin response of endothelial cells *in vitro* thus probably depends on transmethylation events for its full expression. It is also strongly modulated by the intracellular level of cAMP.

Human umbilical vein endothelial cells synthesize the protein component of thromboplastin (apoprotein III) upon treatment with endotoxin, tumor-promoting phorbol esters and phytohaemagglutinin [1]. The response is greatly enhanced in lymphocyte-endothelial cell [1] and platelet-endothelial cell co-cultures [2]. The thromboplastin procoagulant becomes partly available on the cell surface [1]. Levels of procoagulant activity are reached which clearly may be of clinical importance if similar levels appear *in vivo*. The modulation of this response of the endothelial cells therefore becomes important. Pharmacological effects may also contribute to the elucidation of the intracellular mechanisms involved in this response. Based on experience with the corresponding response in human monocytes,†‡ [3] we have investigated the effect of a calcium antagonist (TMB-8),§ two phosphodiesterase inhibitors (MIX and RO-20-1724) and transmethylation inhibitors (DZA and Hcy) separately and in pairwise combinations. The agents used to induce thromboplastin synthesis in endothelial cells were ETX and TPA.

MATERIALS AND METHODS

Chemicals. TMB-8, TPA, MIX, Hcy and human serum albumin were obtained from Sigma, St. Louis, MO. ETX was lipopolysaccharide B (from *E. coli* B4:0111) obtained from Difco, Detroit, MI. DZA was from Southern Research Institute, Birmingham, AL. RO-20-1724 was kindly provided by Hoffmann–La Roche, Basel, Switzerland. For stock solutions, TMB-8 and ETX were dissolved in endotoxin-free RPMI 1640, TPA in aceton, MIX and RO-20-1724 in ethanol and DZA/Hcy in isotonic saline. Each agent was diluted in culture medium to the final concentration desired. A corresponding volume of the appropriate solvent was used in the respective control cultures. Standard and endotoxin-free RPMI 1640 (i.e. <30 pg/ml) were obtained from Gibco Biocult, Paisley, Renfrewshire, Scotland. Foetal calf serum was from Gibco or from Flow, Irvine, Scotland.

Cell cultures. Cultures of human umbilical cord endothelial cells were established and maintained in 24 well cluster trays (Costar, Cambridge, MA) as described previously [1, 4]. The culture medium consisted of standard RPMI 1640 supplemented with 20% foetal calf serum, L-glutamine (300 mg/l) penicillin (100 units/ml), streptomycin (100 μg/ml) and anti-PPLO (5 ml/l). Confluent primary cultures, age 4–10 days, were used for all experiments. Previous experiments have shown that there is no correlation of thromboplastin response with age of culture during this time interval. At the start of each experiment the culture medium was removed and the cells were washed 3 times with standard RPMI 1640. Fresh culture medium (containing either standard or endotoxin-free RPMI 1640) with the appropriate additions was then added and the incubations started. All incubations were performed at 37° in an atmosphere of 5% CO_2 in air. The cells were harvested after 16 hr and washed and lysed as described [1].

Assays. Thromboplastin activity was assayed in a one-stage system using citrated normal human plasma as substrate [5]. Standard curves were es-

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† T. Lyberg and H. Prydz. Unpublished (1983).

‡ Ø. Hetland, A. B. Brovold and H. Prydz. Unpublished (1983).

§ Abbreviations: ETX, endotoxin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TMB-8, 8-(*N*,*N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate hydrochloride; MIX, methylisobutylxanthine; Hcy, 1-homocysteine thiolactone; DZA, 3-deazaadenosine; RO-20-1724, rac-4(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; RPMI 1640, Roswell Park Memorial Institute cell culture medium 1640.

Table 1. Increase of thromboplastin activity in endothelial cells in culture

Stimulant	Thromboplastin activity (units/mg cell protein)	N	Ratio of activity in stimulated cells / control cells
TPA	22.2 ± 12.1	11	5.4
Control	4.1 ± 2.3		
ETX	4.2 ± 0.7	3	5.3
Control	0.8 ± 0.3		

Final concentrations of ETX (endotoxin) and TPA (12-*O*-tetradecanoylphorbol-13-acetate) were 25 μg/ml and 50 ng/ml, respectively. Incubation time, 16 hr.

tablished by diluting a standard preparation of crude human brain thromboplastin [6]. This preparation clotted normal plasma in 14–15 sec and was arbitrarily taken to contain 100 units/ml of thromboplastin activity.

The procoagulant activity observed was due to thromboplastin as evidenced by its sensitivity to phospholipase C, its neutralization by an anti-apoprotein III antibody and its lack of activity in factor VII deficient plasma. These tests were carried out as described previously [1, 7].

Protein was determined by a modification [8] of the Lowry method [9] with human serum albumin (Sigma) as standard. The ^{51}Cr-release assay and the trypan blue exclusion test were performed as previously described [4].

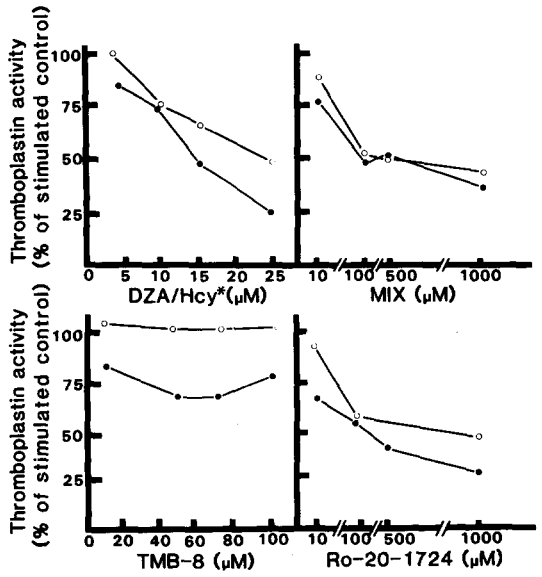

Fig. 1. The effect of various inhibitors on TPA-(●——●) and ETX-induced (○——○) thromboplastin activity in confluent endothelial cell cultures. Endothelial cells were incubated for 16 hr in fresh culture medium to which TPA (50 ng/ml) or ETX (25 μg/ml) was added together with one inhibitor as indicated. Thromboplastin activity and total cell protein were determined. The figures are given as % of the thromboplastin activity induced by TPA (mean 26 units/mg cell protein) or by ETX (mean 5.5 units/mg cell protein) and represent the mean of at least 2 separate experiments (each in duplicate). * The concentration of Hcy was in each case twice that indicated for DZA.

RESULTS

TPA (50 ng/ml) and ETX (25 μg/ml) induced a thromboplastin increase of about 5-fold in the endothelial cell cultures during a 16 hr stimulation period (Table 1) confirming earlier results [1]. The higher background level of thromboplastin in cells stimulated with TPA was caused by small amounts of endotoxin contained in standard RPMI 1640 medium.

The effect of the various inhibitors is illustrated by their dose–response curves (Fig. 1). The calcium blocker TMB-8 had little or no effect on the thromboplastin activity induced by TPA (50 ng/ml) or ETX (25 μg/ml), respectively. The phosphodiesterase inhibitors MIX and RO-20-1724 (both at 1 mM) and the transmethylation inhibitors DZA/Hcy (at 25 μM/50 μM) reduced the ETX-induced thromboplastin response to about 50% (Fig. 1) and the TPA-induced response to about 25–35% (Fig. 1).

TMB-8 (50 μM) was more effective in lowering the TPA-induced thromboplastin activity when tested in combination with MIX (10 or 500 μM) (Fig. 2). The combinations of TMB-8 (50 μM) and DZA/Hcy had additive effect only at the highest concn of DZA/Hcy tested (25 μM/50 μM) (data not shown).

The combination of DZA/Hcy and MIX at various concn induced marked inhibitions of the TPA-stimulated thromboplastin response (Fig. 3).

As judged by phase contrast microscopy, the morphology of the endothelial cells remained normal during incubation with MIX and RO-20-1724. More

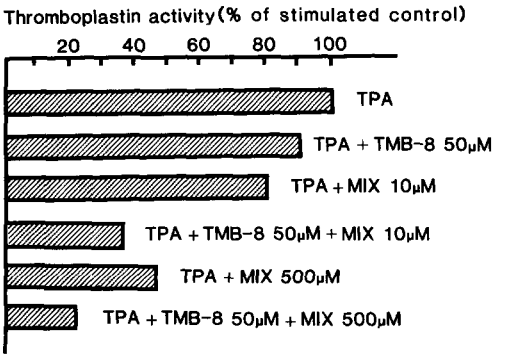

Fig. 2. The effect of TMB-8 and MIX, separately or in combination, on TPA-induced (50 ng/ml) thromboplastin activity (mean 30.7 units/mg cell protein) in confluent endothelial cell cultures. See legend to Fig. 1 for details.

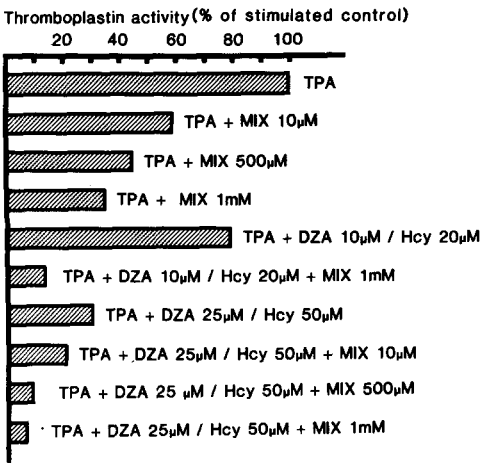

Fig. 3. The effect of MIX and DZA/Hcy, separately or in combination, on TPA-induced (50 ng/ml) thromboplastin activity (mean 19.5 units/mg cell protein) in confluent endothelial cell cultures. See legend to Fig. 1 for details.

than 98% of the cells excluded trypan blue and ^{51}Cr-release was not significantly increased. At the highest concentration used, DZA/Hcy (25 μM/50 μM) and TMB-8 (100 μM) caused minor retraction of endothelial cell borders and detachment of cells. The ^{51}Cr-release increased about 2-fold. Lower concn of these agents gave no microscopic signs of cytotoxicity and no significant increase in ^{51}Cr-release.

DISCUSSION

The aim of this study was 2-fold: (1) Is it possible to influence pharmacologically the thromboplastin synthesis induced in endothelial cells? (2) Can these pharmacological effects give us information about the intracellular mechanisms involved in the response?

MIX and RO-20-1724 are well established inhibitors of phosphodiesterases, thus increasing intracellular levels of cAMP. The fact that both inhibitors reduced the TPA- as well as the ETX-induced thromboplastin synthesis suggest that the phosphodiesterase activities are important modulators of the thromboplastin response. The effect of the phosphodiesterase inhibitors is probably mediated by enhanced intracellular levels of cAMP as addition of exogenous cAMP (dbcAMP) inhibited the thromboplastin response to an equal degree (unpublished observations). This is also in accordance with results obtained with human monocytes where phosphodiesterase inhibitors, adenylate cyclase stimulators and cAMP analogues all inhibit induction of thromboplastin synthesis [3] but other interpretations are not entirely excluded since phosphodiesterase inhibitors have other effects [10–14]. The inhibitory effect of these phosphodiesterase-inhibitors on TPA-induced thromboplastin synthesis in monocytes is, however, much less pronounced than in endothelial cells. The effect on ETX-induced thromboplastin synthesis is about equal in the two cell types.

On a molar basis, the transmethylation inhibitors were the most effective inhibitors of thromboplastin synthesis in endothelial cells. Furthermore, DZA/Hcy in combination with MIX, extinguished the thromboplastin response to TPA. These observations suggest that transmethylation is important in the induction of thromboplastin synthesis in the endothelium. This interpretation is supported by recent findings of Hetland *et al.* (unpublished), who showed that DZA and Hcy together prevented the induction of thromboplastin synthesis in human monocyte cultures. However, at the highest concentration used these compounds gave cytotoxic effects. The inhibitory effect at this concentration may therefore be rather unspecific. At lower concentrations where no cytotoxic effects were observed, there was also a significant inhibitory effect of DZA/Hcy. The transmethylation reactions involved are so far unknown. The transmethylation reactions involved are so far unknown. Most likely protein or phospholipid methylation is involved.

The expression of thromboplastin activity may involve phospholipid rearrangement, since the thromboplastin protein requires a mixture of phospholipids, including some with negative charge, for optimum activity [15, 16]. Negatively charged phospholipids are usually scarce in the outer leaflet and rearrangements from the inner leaflet may be necessary.

TMB-8 had no or very slight inhibitory effect on induction of endothelial cell thromboplastin. However, together with MIX, TMB-8 inhibited the thromboplastin response, thus suggesting that intracellular Ca^{2+} may participate as a regulating factor for thromboplastin synthesis when cAMP is increased. A role for Ca^{2+} in the thromboplastin response of monocytes has recently been suggested (Lyberg and Prydz, unpublished).

We conclude that the elicitation of thromboplastin synthesis in cultured endothelial cells exposed to TPA depends on transmethylation reaction(s) which so far remain undefined and on phosphodiesterase activity most likely mediated via its effect on cAMP levels. The thromboplastin response of endothelial cells to ETX is slightly less sensitive to the phosphodiesterase and transmethylation inhibitors and insensitive to the Ca^{2+} blocker.

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