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ACTIVATION OF S_6 KINASE IN CULTURED VASCULAR SMOOTH MUSCLE CELLS BY SUBMITOGENIC LEVELS OF THROMBOSPONDIN

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SUMMARY. Purified human platelet thrombospondin was shown to activate S_6 kinase in cultured vascular smooth muscle cells in a dose- (1-9 µg/ml) and time-dependent manner. Down regulation of epidermal growth factor and somatomedin C receptors by prior treatment of cells with their respective growth factors did not reduce this effect. Kinase activation by thrombospondin was only marginally reduced in the presence of platelet-derived growth factor specific antibody at levels that totally inhibited platelet-derived growth factor (5 ng/ml) induced activation. Additionally, thrombospondin elicits a rapid dose-dependent phosphoinositide turnover response analogous to that of platelet-derived growth factor, epidermal growth factor and somatomedin C. Prior treatment of cells with phorbol ester for 48 hrs in serum-free culture medium resulted in a small enhancement of S_6 kinase activation by thrombospondin and the above mentioned growth factors but a complete loss in the ability of phorbol ester to activate this enzyme. These findings with cultured smooth muscle cells suggest a growth factor-like role for thrombospondin.

INTRODUCTION. The process of cellular proliferation is intimately associated with the protein synthetic status of the cell. Initiation/activation of protein synthesis is a prerequisite for cells to transit from the quiescent to the proliferative state (1-4). Part of this activation process involves the multiple phosphorylation of an integral 40S ribosomal subunit polypeptide, S_6. This event leads to the formation of initiation complexes between messenger RNA and mono-(80s) ribosomes and subsequently actively translating polysomes (5,6). While a number of protein kinases have been implicated in this process (7,8), there is increasing evidence that in vivo this process in controlled by a specific soluble-cytoplasmic kinase system termed S_6 kinase (9,10), which itself is also subject to activational control via phosphorylation. Stimula-

Abbreviations: VSMC, vascular smooth muscle cells; hTS, human thrombospondin; FCS, fetal calf serum; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF_1, somatomedin C; SHR, spontaneously hypertensive rat; IP_3, inositol trisphosphate; PIP_2 phosphatidylinositol bisphosphate; PMA, phorbol 12-myristate 13-acetate; MEM, minimal essential medium; PBS, phosphate buffered saline.

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tion of S_6 kinase *in vivo* has been shown to occur rapidly in response to growth factor-receptor interaction (10,2,11).

Recent observations have suggested an important role for epidermal growth factor (EGF) , in relation to vascular smooth muscle cell (VSMC) metabolism (12,13). EGF is generally considered to be a progressive factor for VSMC but has been shown to be mitogenic to epidermal cells (14). However, in the presence of low levels of serum or the platelet-derived glycoprotein thrombospondin (TS) EGF becomes 'upgraded' to a mitogen for cultured VSMC (12). This glycoprotein which belongs to the growing group of matrix associated large molecular weight glycoproteins was first characterized from human platelets (15). Subsequently cells of mesenchymal origin have been shown to be capable of synthesizing and incorporating TS into their extracellular matrix (16,17). Such synthesis may be rapidly stimulated by brief exposure of quiescent cells to platelet-derived growth factor (PDGF) (18). Although TS even at high doses is only mildly mitogenic to VSMC, low levels of this glycoprotein when combined with EGF elicited a mitogenic response comparable to that obtained with PDGF, an acknowledged mitogen for VSMC (12).

We report here on our studies using purified human platelet thrombospondin (hTS) at submitogenic levels and its effects on intracellular metabolic events usually associated with proliferating cells.

MATERIALS AND METHODS

Materials. All tissue culture material and chemicals were obtained from Gibco AG, Basel, Switzerland except for fetal calf serum (FCS) which was purchased from Fakola AG, Switzerland. Anti-human platelet-derived growth factor immunoglobulin G (anti-PDGF) was obtained from Collaborative Research, Bedford, USA as were PDGF and EGF. Somatomedin C (insulin-like growth factor 1; IGF_1) and all radioactive isotopes were supplied by Amersham, Zürich, Switzerland and included $[\gamma^{32}P]$-ATP (3000 Ci/mmol), Myo-[2-^{3}H]inositol (16-20 Ci/mmol), [methyl-^{3}H]-Thymidine (92 Ci/mmol); Phorbol 12-myristate 13-acetate (PMA) was from Sigma Chemical Co, St. Louis, USA. Lab. Tek. eight chamber slides were supplied through Bayer AG (Miles), Zürich, Switzerland. All other chemicals and reagents were obtained either from E. Merck, Darmstadt, FRG or Sigma Chemical Co., St. Louis, USA.

Methods. VSMC from thoracic aortas of SHR rats were isolated using procedures described previously (19). SHR animals were used since a greater yield of fast growing cells (vs control rats) could be obtained. Cultures were used between 5th and 14th passage and cell numbers determined following enzymatic disaggregation of cell layers (20). Cells were maintained in MEM containing Earles salts, 20 mM glutamine, 20 mM TES-NaOH, 20 mM HEPES-NaOH (both pH 7.3), and 100 U/ml penicillin and 100 U/ml streptomycin as bacteriostatic agents. Media was supplemented with 10% v/v heat-inactivated fetal calf serum except for the 48 hr period prior to assays involving S_6 kinase activation or nuclear labelling (see below).

Calcium-replete human platelet thrombospondin (hTS) was prepared from fresh whole blood essentially as described by others except that platelet aggregation was induced using Ionomycin (12,21). Purity was assessed by analysis of overloaded samples on SDS/ PAGE. Final protein concentrations ($E^{\%}_{1cm}$ = 10.9 (21)) ranged from 350-900 µg/ml. Samples were finally dialysed against PBS containing 0.5 mM $CaCl_2$ and stored in aliquots at -20°C.

The activation of S_6 kinase in cells maintained on serum free medium for 48 hrs was performed essentially as described previously with the following modifications (10). Cells were plated into 6 well multiwell plates at $2x10^5$ cells/ well and grown to confluency in normal media. Thereafter, medium was replaced with serum-free media and appropriate additions as indicated in Results. After 48 hrs the latter culture medium was replaced with fresh serum-free containing hTS and other additives as specified (Results), and then cells were incubated at 37°C for the required periods. At the end of activation incubations plates were washed with 3x 2 ml aliquots of cold extraction buffer (EB: 20 mM HEPES, 15 mM $MgCl_2$, 20 mM EGTA, 1 mM DTT and 80 mM β-glycerol phosphate, pH 7.3). Cell layers were scraped into microcentrifuge tubes with 300 µl of EB containing 0.2% v/v Triton x-100 and 5 mM PMSF, and lysates collected following centrifugation for 15 min at 11,000 g at 5°C. Samples were stored at -70°C until required for S_6 kinase phosphorylation assays. This modified extraction procedure yielded identical data to that obtained using the method published previously (10). S_6 kinase phosphorylation assays were performed using 40S ribosomal subunits as substrate essentially as described previously (10) (specific activity of [γ^{32}P]-ATP at 2×10^4 dpm/pmol). Based on extractions of cell layers having between 1.0 and $3.2x10^6$ cells/ well, assays were linear with respect to time and protein concentration and 40S subunits were used at saturation levels (10). Stimulation of cells with 10% FCS resulted in the incorporation of between 4 and 4.65 pmol $PO_4/10^6$ cells into S_6 polypeptide and unstimulated cells (serum-free) exhibited values of 0.42 to 0.48 pmol $PO_4/10^6$ cells. These values were obtained from ± 120 phosphorylation assays.

To study phosphoinositide metabolism confluent cells were prelabelled for 36 hrs with myo[2-^{3}H]-inositol (5 µCi/ml) in serum-free medium (22). Thereafter, cells were washed with PBS prior to addition of 1 ml isotonic PBS containing 50 mM LiCl. Cells were preincubated for 30 min at 37°C before exposure to hTS. Termination of incubations and fractionation of phosphoinositides and inositol phosphates was performed as previously described (22, 23).

Mitogenesis assays (nuclear labelling) was performed using VSMC plated onto Lab. Tek. eight chamber slides rinsed with 1% v/v gelatine (24). Sparsley confluent cultures were maintained on serum-free media (with one change) for 48 hrs. Quiescent cells were exposed to hTS and 10% v/v FCS in serum-free medium for 28 hrs in the presence of methyl [^{3}H]-thymidine (1 µCi/ml). Thereafter, cells were fixed and slides processed described (12,19).

Unless stated otherwise all values in the text are given as mean ± S.D., where n is the number of separate experiments. Statistical analysis was performed using Student's t-test for unpaired data when appropriate.

RESULTS AND DISCUSSION. Exposure of quiescent VSMC to hTS (see Fig. 1) resulted in a dose-dependent activation of S_6 kinase as evidenced by the increased ability of lysates from treated cells to incorporate ^{32}P into the S_6 polypeptide of 40S subunits. hTS-induced activation reached maximum levels at glycoprotein concentrations of ~1.5 µm. These low levels of hTS were found to be non-mitogenic in nuclear labelling mitogensis assays (% total nuclei labelled for serum-free = 8.1 ± 1.7%; for hTS (9 µg/ml) = 7.9 ± 1.9%; for 10% v/v FCS = 78.4 ± 5.9%) which is in agreement with the findings of others (12).

Purified hTS preparations showed a single polypeptide band (Mr 180 KDa) on SDS-polyacrylamide gels (Fig. 2) with no visible evidence of smaller molecular weight components. It is noteworthy that other preparations of hTS, which were not used in these studies because of evidence for proteolytic degradation (3 bands on SDS-polyacrylamide gels with molecular weights of Mr of 140 KDa, 120

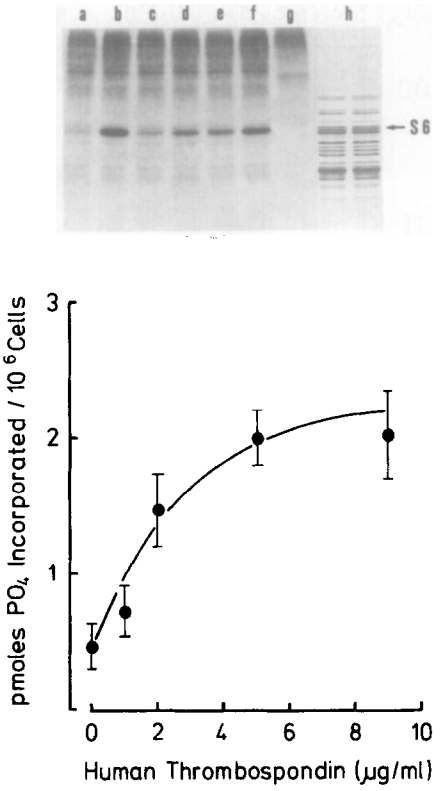

Fig. 1. Dose-dependent activation of S_6 kinase in VSMC by hTS. Activation of quiescent cells and subsequent phosphorylation assays performed on kinase extracts were as described in Methods. Superimposed above the dose response curve is a typical autoradiograph obtained from 17.5% SDS- polyacrylamide gels as used in phosphorylation assays (Methods). The lanes represent samples from VSMC exposed to a) serum-free medium, b) 10% FCS medium, c-f) levels of hTS as given in the figure below, g) the same as b) but assayed in absence of 40S substrate and h) two coomassie blue stained lanes typical of the protein profiles obtained in our procedure.

KDa and 25 KDa), were capable of activating S_6 kinase to a degree comparable with the single band preparations suggesting that this biological activity resides in one or more proteolytically stable domains like that for type V collagen binding (25).

PGDF, EGF and IGF_1 all induce S_6 kinase activation at concentrations (nM) almost 1000-fold lower than hTS (µM). Therefore, in spite of the apparent purity (> 95%) of hTS preparations it was deemed necessary to ensure that activation of S_6 kinase by glycoprotein isolates was due to hTS specifically and not an artifact of contamination with growth factors at levels sufficient for activation but undetectable on SDS-polyacrylamide gels. The dose-dependent activation of kinase by hTS was only marginally reduced when glycoprotein preparations were treated with specific monoclonal antibodies to PDGF at levels sufficient to completely abolish enzyme activation by 5 ng of PDGF (Table 1). The profile obtained for the dose-response of VSMC to hTS was also

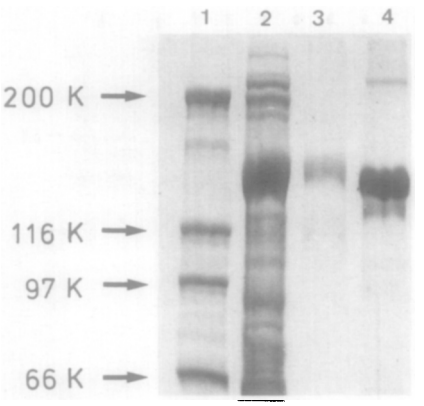

Fig. 2. SDS-PAGE analysis of hTS. Samples obtained from the supernatant after platelet aggregation (lane 2), Heparin-Sepharose CL-6B affinity chromatography (lane 3) and the same overloaded (lane 4) were analysed on 5% SDS-polyacrylamide gels. The molecular weight markers (lane 4) used were myosin (200 k), β-galactosidase (116 k), phosphorylase B (97.4 k) and bovine serum albumin (66.2 k).

inconsistent with low levels of contamination by PDGF (and EGF or IGF_1) since S_6 kinase activation by glycoprotein preparations reached maximum levels that were markedly lower (2.14±0.27 pmol $PO_4/10^6$ cells) than the possible maximum levels ($\sim$3 pmol $PO_4/10^6$ cells; see Table 1) for these growth factors (Fig. 1).

The kinetics of activation of S_6 kinase by hTS were slow compared to EGF (Fig. 3) and, unlike the latter, activation by hTS was persistent rather than transient (Fig. 3). A similar sustained slow response has been reported for orthovanadate S_6 kinase activation in 3T3 cells (10). A progressive loss in the ability of EGF to activate S_6 kinase was observed both in the absence or presence of hTS (Fig. 3; Table 1) and can be attributed to 'down regulation' of EGF receptors (19). However, in the presence of hTS activation levels remained at those observed for hTS alone at times (> 80 mins) when EGF (alone) showed markedly reduced ability to perform such a task (Fig. 3). Analogous pretreatment experiments with IGF_1 yielded similar results in that VSMC S_6 kinase became passive to subsequent IGF_1 exposure but maintained a normal activation response to hTS (Table 1).

All these findings together with the aforementioned complete absence of mitogenic activity in glycoprotein preparations provide support that the observed S_6 kinase activation in VSMC by these preparations was due to hTS alone and not the result of contamination by PDGF, EGF or IGF_1.

The activation of S_6 kinase is intimately linked to the operation of phosphorylation pathways controlled by two distinct protein kinases, namely tyrosine kinase (26) and protein kinase C (PkC) (27). The activation of PkC by growth factors is dependent upon production of diacylglycerol via agonist-receptor stimulated phosphoinositide catabolism (28). Tumour promoting phorbol

Table 1

Effect of various VSMC pretreatments on subsequent S_6 kinase activation levels

Pretreatment	Activation/Treatment	pmol PO_4 Incorporated/10^6 cells
None	PDGF	3.20 ± 0.14
Anti PDGF	PDGF	0.65 ± 0.07
Anti PDGF	hTS	1.83 ± 0.19
None	IGF_1	2.46 ± 0.21
IGF_1	IGF_1	0.87 ± 0.11
IGF_1	hTS	1.64 ± 0.20
None	EGF	3.36 ± 0.27
EGF	EGF	1.10 ± 0.10
EGF	hTS	1.90 ± 0.20
None	PMA	3.57 ± 0.40
TPA	PMA	0.63 ± 0.12
TPA	hTS	2.10 ± 0.23

Cultures of VSMC were pretreated with the following agents prior to exposure to either the same agent or hTS (4.5 µg/ml) as indicated below for 20 min. All pretreatments were performed with the agents in serum-free medium on cultures maintained for 48 hrs on such medium except in the case of PMA where the pretreatment period encompassed the 48 hrs on serum-free medium. Agents used for both pretreatment and S_6 kinase activation included PDGF (5 ng/ml), human anti PDGF (10 µg simultaneously with PDGF addition), IGF_1 (5 ng/ml, 12 hrs pretreatment), EGF (5 ng/ml, 4 hrs pretreatment) and PMA (100 nM, 48 hrs pretreatment). The data express pmole PO_4 incorporated per 10^6 cells; values for FCS, serum-free and hTS (no pretreatment) were 4.2 ± 0.27, 0.46 ± 0.08, 1.95 ± 0.18 pmoles PO_4 incorporated, respectively.

esters (PMA), which are diacylglycerol mimetics also activate PkC (28) with attendant activation of S_6 kinase.

In order to ascertain whether the PkC phosphorylation pathway might be involved in hTS-dependent S_6 kinase activation the influence of hTS on VSMC phosphoinositide metabolism was studied. The time- and dose-dependent accumulation of $InsP_3$ and concomitant loss of Ptd-$InsP_2$ in VSMC exposed to hTS (Fig. 4) indicated that this glycoprotein can elicit stimulation of phosphoinositide turnover, and thus by inference, activation of PkC (29).

Prolonged (48 hrs) pretreatment of VSMC with low PMA doses has been shown to negate any subsequent activation of PkC (30). While such prolonged exposure to PMA rendered VSMC S_6 kinase completely passive to subsequent exposure to

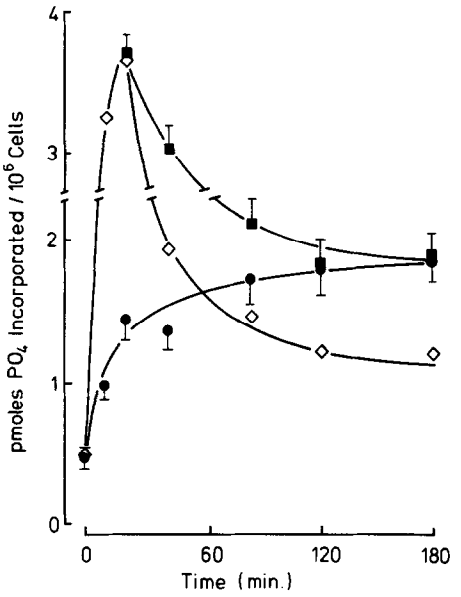

Fig. 3. Time course for the activation of S_6 kinase in quiescent VSMC by hTS (4.5 µg/ml, ●), EGF (5 ng/ml, ◆) and EGF + hTS together (5 ng EGF + 4.5 µg hTS, ■). Activation and phosphorylation assays were performed as described under Methods and the data for EGF represents the means of complete duplicate assays (activation and phosphorylation) which were repeated only once since our finding agreed with many previously reported similar experiments (10, inter alia). For hTS and EGF + hTS data represents means ± SD (n=5 and 3, respectively).

PMA, it did not reduce the ability of hTS to elicit activation of VSMC S_6 kinase (Table 1). This pretreatment also did not affect S_6 kinase activation responses to EGF, PDGF, and IGF_1 (data not shown). Since tyrosine kinase activity is intrinsically associated with EGF, PDGF and IGF_1 receptors (14,25) these findings invoke the possible involvement of this phosphorylation pathway in hTS-induced S_6 kinase activation. Positive identification of specific TS-receptors on VSMC is required before this proposal can be pursued. Current investigations on the binding of [I^{125}]-hTS to intact VSMC have been hampered by high non-specific binding which might relate to the known ability of TS to become deposited in the matrix of VSMC (12).

Recently it has been reported that there are similarities between the superinduction of TS mRNA in response to PDGF in the presence of cycloheximide and that of the C fos oncogene mRNA (31). These findings would suggest that TS may be more important in the control process of proliferation than previously envisaged. We feel that one important physiological consequence of S_6 kinase activation by TS would be to stimulate cellular protein synthetic capacity such that VSMC exposed to TS transit to a permissive state which allows them to proliferate in response to low levels of mitogenic compounds (12). We are currently investigating some aspects relating to protein synthesis such as

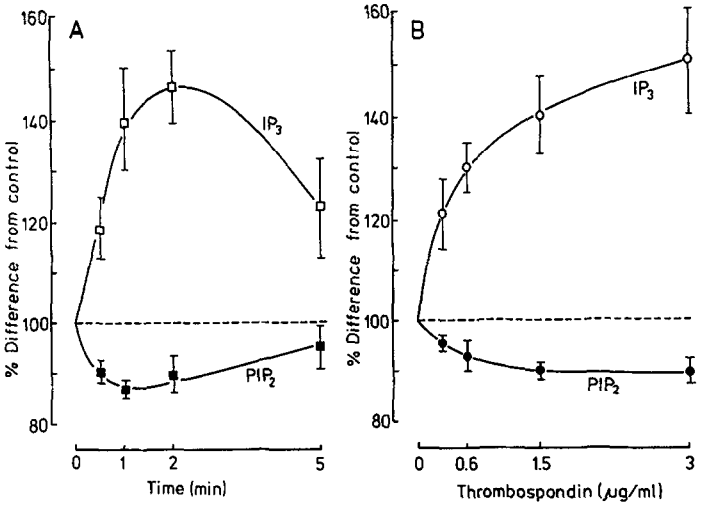

Fig. 4. Thombospondin-stimulated phosphoinositide breakdown in VSMC. [^{3}H]myo-inositol prelabelled VSMC were exposed to 1.5 µg hTS/ml for various times (panel A) or to various concentrations of hTS for 90 sec (panel B) in the presence of LiCl. [^{3}H]-content of IP_3 (○, □) and PIP_2 (after deacylation, ●, ■) was determined following extraction and separation by ion exchange chromatography. Experimental details are described in Materials and Methods. Values (mean ± SD, n=4) express change (%) in [^{3}H]-content relative to control values (100%).

recruitment of phosphorylated 40S subunits into polysomes as a consequence of hTS interaction with VSMC.

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