Effect of Thrombin, the Thrombin Receptor Activation Peptide, and Other Mitogens on Vascular Smooth Muscle Cell Urokinase Receptor mRNA Levels

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Bovine vascular smooth muscle cells (SMC) express the urokinase-type plasminogen activator receptor (u-PAR) claimed to be important in cell invasion. Receptor numbers and affinity are regulated by thrombin and several other mitogens involved in SMC proliferation. We investigated the effects of these mitogens on u-PAR mRNA levels. On continuous thrombin stimulation the u-PAR message in SMC was 10 ± 2.3-fold elevated reaching a maximum between 6 and 9 hours and declining to control values within 48 hours. Thrombin present for 30 minutes on the cell surface produced similar effects. Stimulation with the thrombin receptor activation peptide S-F-L-L-R-N representing the NH2-terminus of the tethered ligand also increased u-PAR mRNA levels with an identical time course. D-Phe-Pro-Arg-chloromethyl ketone (PPACK) active site blocked thrombin and the catalytically inactive thrombin mutant S205A did not affect u-PAR mRNA levels. Thrombin stimulation also resulted in a 2 ± 0.2-fold transient increase in thrombin receptor mRNA preceding the rise in u-PAR message. Transforming growth factor β1 (TGFβ1) and platelet-derived growth factor (PDGF) showed similar time courses for the elevation of u-PAR mRNA levels with a maximal 5.5 ± 0.9 and 12 ± 2.5-fold increase, respectively. Basic fibroblast growth factor (bFGF) and phorbol myristate acetate (PMA) showed a more prolonged effect increasing u-PAR mRNA levels 8 ± 2.0-fold and 12.3 ± 2.5-fold, respectively, within 6 hours but remaining 5 to 10-fold elevated at 48 hours. In order to decide if the u-PAR mRNA increase was due to message stabilization or a consequence of transcriptional activation we used the RNA polymerase II inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) during the stimulation experiments. u-PAR mRNA levels on TGFβ1 stimulation of SMC decayed after the addition of DRB indicating that enhancement of transcriptional activity was involved in the induction. In contrast, the time course of u-PAR mRNA elevation on thrombin, bFGF, and PMA stimulation was not significantly altered in the presence of DRB suggesting that in these latter cases u-PAR mRNA message accumulation was at least in part due to mRNA stabilization. Increased transcriptional activity, mRNA stabilization and expression of u-PAR protein on the SMC surface in response to growth factors may facilitate enhanced cell surface protease activity, cell migration, and development of atherosclerotic lesions.

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MATERIALS AND METHODS

Materials. Bovine and human α-thrombins (2,800 National Institute of Health [NIH] U/mg), the thrombin mutant S205A, the thrombin receptor activation peptide (TRAP: S-F-L-L-R-N), human platelet-derived growth factor (PDGF: BB-homodimer), human transforming growth factor β1 (TGFβ1), human recombinant basic fibroblast growth factor (bFGF), phorbol myristate acetate (PMA), bovine pancreas insulin, bovine transferrin, cycloheximide, D-Phe-Pro-Arg-chloromethyl ketone (PPACK), tissue culture reagents and dishes were obtained from the vendors or colleagues previously listed. Restriction enzymes, T4-DNA-ligase, T4-DNA-kinase, T4-DNA-polymerase, G50-Sephadex Quick spin columns for DNA and RNA were purchased from Boehringer Mannheim, Indianapolis, IN. An in vitro transcription kit, pGEM-vectors, a cell proliferation assay, and an RNA isolation kit were from Promega, Madison, WI. An RNAse protection assay kit was from Ambion, Austin, TX. [32P]-UTP was obtained from NEN Dupont, Boston, MA. A commercial Superscript preamplification kit for first strand cDNA synthesis, an Escherichia coli competent host cell strain DH5-α (BRL, Gaithersburg, MD), a plasmid isolation kit (Qiagen, Chlotsworth, CA) and reagents for polymerase chain reaction (PCR) (Perkin Elmer Cetus, Norwalk, CT) were used throughout. Synthesis and purification of PCR primers were performed at Northwestern University, Chicago.

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IL. DNA purification and sequencing were done with a Prep-A-Gene kit (BioRad, Richmond, CA) and a DNA Sequenase kit (United States Biochemicals, Cleveland, OH), respectively. TRIS-borate-EDTA (TBE) 5% polyacrylamide/6 mol/L urea gels were purchased from Enprotech, Natick, MA. 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) and actinomycin D were from Sigma, St Louis, MO. Molecular weight standards (Lambda-DNA-HindIII-Phix174-DNA-HaelII digest) were from Pharmacia, Alameda, CA. Films for autoradiography were purchased from Amersham, Arlington Heights, IL.

Cell culture. SMC were isolated from bovine aortas by the explant technique and cultivated as recently described. All stimulation experiments were performed in cell passage 2-4. Confluent cultures were washed twice in growth arrest medium (Dulbecco’s Modified Eagle Medium, 5 mg/mL bovine insulin, and 5.6 mg/mL bovine transferrin) and then grown arrested for 48 to 72 hours before stimulation experiments. Stimulation at 37°C was performed for different time periods with the following mitogen concentrations: 40 U/mL thrombin, 50 μg/mL TRAP, 200 μg/mL PMA, 20 ng/mL PDGF-BB, 10 ng/mL bFGF, 10 ng/mL TGF-β1, and 10 μg/mL cycloheximide. Mitogen concentrations were selected based on dose responses for u-PAR protein expression. Cell viability was determined by trypan blue exclusion and cell proliferation assays according to the manufacturer’s recommendations.

Treatment of SMC with DRB. SMC were prestimulated for 3 hours with the different mitogens as described above followed by the addition of the RNA-polymerase inhibitor DRB (25 μg/mL) to the culture supernatants. At distinct time intervals after the addition of DRB, the cells were lysed in guanidinium thiocyanate and the lysates stored at −80°C for total RNA isolation. As a control, SMC were treated with DRB alone to analyze the signal consistency of the control gene APP over the observed time span. In addition SMC were pretreated for 2 hours with DRB and thereafter stimulated with TGF-β1, bFGF, PMA and thrombin to prove that DRB efficiently blocked transcription within that experimental setting.

RNA isolation. Total RNA was isolated from growth arrested and stimulated SMC by acid guanidinium thiocyanate-phenol-chloroform extraction.

Isolation of cDNA clones and subcloning. A full-length cDNA clone for bovine u-PAR was isolated from a bovine aorta endothelial cell (BAEC) cDNA library. A 279 bp u-PAR cDNA fragment was excised from Bluescript SK− with Pst I and Xho I (166-445 bp) and directionally subcloned into the Pst I and Xho I sites of pGEM-3Z. The thrombin receptor clone was isolated from the same BAEC cDNA library and a 452-bp Kpn I Ava II fragment subcloned into the Smal restriction site of the pGEM-7Z vector. As an internal standard for equal loading of RNA in RNase protection assays, an internal standard, a 229 bp fragment of the bovine β-amylod precursors protein (APP) was generated by PCR using the following primers: APP 5’ : GGT TGA CAA ATA TCA AGA CCG and APP 3’ : TGA TGA ATG GAT GTG TAC TG. A bovine c-myc fragment (314 bp) was generated by PCR applying the following primers: 5’ : CCA GTG ACC ACA AGC and 3’ : CTG TGG TGT AAG TGC. Bovine cDNA, which was reverse transcribed from bovine total SMC RNA, served as a template for PCR. The PCR products were subsequently labeled with 50 U/mL of T4-DNA-polimerase and 50 U/mL of T4-DNA-kinase, and ligated into the Sma I restriction site of the pGEM-3 and pGEM-4Z transcription vectors, respectively. Transformation was performed using the competent E. coli host cell strain DH5α. Bacteria were amplified in 2 × yeast tricthrome containing 50 μg/mL ampicillin. The plasmid was isolated using the reagents and the protocol supplied by the manufacturer. To allow in vitro transcription of suitable anti-sense cRNA, the u-PAR template was linearized with HindIII, the thrombin receptor template with Xba I, and the APP template with BamHI. Template DNA was then gel purified on 1% agarose/TBE gels using the Prep-A-Gene kit and subsequently in vitro transcribed using the T7- or Sp6-RNA polymerase.

In vitro transcription. The in vitro transcription was performed according to the manufacturer’s recommendations except that 50 μCi of [32P]UTP at 400 Ci/mmole were used for radiolabeling instead of [32P]UTP and no unlabeled UTP was added. After transcription for 60 minutes at 37°C, the DNA template was removed by digestion with 1 U RNase-free DNase, and unincorporated [32P]UTP eliminated by size fractionation over a G50-Sephadex column for RNA. The efficiency of incorporation of [32P]UTP into the cRNA was quantified in a Beckmann liquid scintillation counter.

RNase protection assay. RNase protection assays were performed according to the manufacturer’s recommendations with some modifications: 5 to 20 μg of total RNA were incubated with [32P]-labeled cRNA probes in hybridization buffer (80% denized formamide, 100 mmol/L sodium citrate, pH 6.4, 300 mmol/L sodium acetate, pH 6.4, and 1 mmol/L EDTA), heated for 3 to 4 minutes at 95°C and hybridized overnight at 42°C in a water bath. Thereafter, 5 U/mL of RNase A and 200 U/mL of RNase T1 were added to the hybridization mixtures and incubated for 30 minutes at 37°C. The RNase treatment was stopped and the protected fragments precipitated by adding 300 μL of RNase inactivation/precipitation buffer. After 2 hours of precipitation at −20°C, the solutions were centrifuged at 10,000 × g for 15 minutes at 4°C and the supernatants decanted. The protected fragments were resuspended, electrophoresed on TBE-polyacrylamide/urea gels, dried, and exposed to x-ray films at −80°C. The band intensity on autoradiographs was quantitatively analyzed by densitometry (Ultrascan XL Laser Densitometer, Pharmacia, Piscataway, NJ). The relative abundance of the u-PAR and thrombin receptor mRNA was determined by normalizing the integrated signal from the protected fragments of interest to the integrated signal of the internal standard APP. Alternatively, original gels were scanned in a β-scanner (Betascope 603 Blot Analyzer, Betagen, Mountain View, CA) and the relative abundance of each mRNA estimated by the ratio between the radioactivity of the signal of interest and the signal from the internal standard.

RESULTS

Effect of thrombin on u-PAR mRNA levels. On continuous thrombin stimulation, u-PAR mRNA levels were increased in a time-dependent fashion reaching a maximum of 10 ± 2.3-fold elevation over those from total RNA of unstimulated SMC. u-PAR message levels were maximally elevated within 6 to 9 hours and declined to control values within 48 hours (Fig 1). Identical u-PAR mRNA increases were obtained when thrombin exposure to SMC was limited to 30 minutes. Unstimulated control SMC showed unchanged mRNA levels over 48 hours. The specificity of the response of SMC to thrombin was studied using catalytically inactive thrombins. SMC did not respond with an elevation of u-PAR mRNA levels when active site titrated PPACK-thrombin or the inactive thrombin mutant S205A were used. In these cases, the hybridization signals were identical to those of unstimulated controls (Fig 2). Stimulation with TRAP (S-F-L-L-R-N, 50 μmol/L) resulted in identical u-PAR mRNA accumulation (Fig 3). Thrombin stimulation was also accompanied by a 2 ± 0.2-fold transient upregulation of thrombin receptor mRNA within 60 minutes, preceding the rise of the u-PAR message (Fig 4).

To examine if de novo protein synthesis was involved in the increases in u-PAR message produced by thrombin we...
Fig 1. Time course of the effect of thrombin on u-PAR mRNA levels in bovine vascular SMC. Bovine vascular SMC were exposed to thrombin (40 U/mL) and the cells harvested in guanidinium thiocyanate solution at time point “0” (lane 1), 15 minutes (lane 2), 30 minutes (lane 3), 1 hour (lane 4), 2 hours (lane 5), 4 hours (lane 6), 6 hours (lane 7), 9 hours (lane 8), 12 hours (lane 9), 24 hours (lane 10), and 48 hours (lane 11). RNA isolation and RNase protection assays were performed as described under Materials and Methods. 2.5 × 10^6 cpm of [32P]-labeled u-PAR antisense cRNA probe and 2 × 10^3 cpm of the [32P]-labeled APP antisense cRNA probe were co-hybridized with 5 μg of total SMC RNA overnight at 42°C. The protected fragments (u-PAR: 279 bp; APP: 229 bp) were electrophoresed on TBE/polyacrylamide urea gels, which were dried and exposed to x-ray films for autoradiography. M: [32P]-labeled Lambda HindIII Phi X Hae III DNA markers.

Fig 2. Effect of thrombin, PPACK-thrombin, and the thrombin mutant S205A on u-PAR mRNA levels in bovine vascular SMC. Bovine vascular SMC were incubated for 4, 6, and 12 hours with thrombin (THR: 40 U/mL), equal concentration of PPACK-thrombin (PPACK-Th), or the thrombin mutant S205A. Five micrograms of total SMC RNA were hybridized overnight with 2 × 10^6 cpm of [32P]-labeled u-PAR cRNA probe and 2 × 10^3 cpm of the internal standard APP at 42°C. RNase protection assays were performed as described under Materials and Methods. The signal intensity on autoradiographs obtained by the u-PAR probe was quantified using a laser densitometer and normalized against the signal from the internal APP standard. u-PAR mRNA levels of unstimulated controls (--) were set equal to “1” and the relative abundance of u-PAR mRNA expressed as “fold induction” at different time points of stimulation. Each value represents the average of triplicate experiments.

Fig 3. Time course of the effect of the thrombin receptor activation peptide on u-PAR mRNA levels in bovine vascular SMC. Bovine vascular SMC were exposed to TRAP (50 μmol/L) and the cells harvested in guanidinium thiocyanate solution at time point “0” (lane 1), 30 minutes (lane 2), 1 hour (lane 3), 2 hours (lane 4), 4 hours (lane 5), 6 hours (lane 6), 9 hours (lane 7), 12 hours (lane 8), 24 hours (lane 9), and 48 hours (lane 10). RNA isolation and RNase protection assays were performed as described under Materials and Methods. 2.5 × 10^6 cpm of [32P]-labeled u-PAR antisense cRNA probe and 2 × 10^3 cpm of the [32P]-labeled APP antisense cRNA probe (lane 11) were cohybridized with 5 μg of total SMC RNA overnight at 42°C. The protected fragments (u-PAR: 279 bp; APP: 229 bp) were electrophoresed on TBE/urea gels, which were dried and exposed to x-ray films for autoradiography. M: [32P]-labeled Lambda HindIII Phi X Hae III DNA markers.

treated SMC with cycloheximide alone that resulted in increased u-PAR mRNA levels in a time-dependent fashion 3 to 5-fold with steady state levels at 24 hours. SMC exposed to thrombin in combination with cycloheximide showed an additive effect on u-PAR message levels and no decline was noted within 24 to 48 hours as noted on thrombin stimulation (data not shown).

Other mitogens/growth factors. Mitogens shown to exhibit a significant effect on u-PAR protein (receptor numbers and affinity) were studied with respect to their effect on u-PAR mRNA. TGFβ3, and PDGF both increased u-PAR message levels to a maximum of 5.5 ± 0.9 and 12 ± 2.5-fold elevation, respectively, within 6 to 9 hours and returned to basal mRNA levels within 24 to 48 hours in exact parallel with thrombin stimulation. Basic FGF stimulated u-PAR mRNA abundance in a more prolonged fashion. Maximal 8 ± 2.0-fold elevation occurred within 6 hours and a steady 5 to 10-fold elevation over controls was noted up to 48 hours. Like thrombin, bFGF elicited a 2 ± 0.2-fold transient upregulation of the thrombin receptor message with a maximum at approximately 1 hour (data not shown). When stimulation experiments were performed with thrombin in combination with either PDGF-BB, TGFβ3, or bFGF, an additive effect was observed with 15- to 18-fold increased u-PAR mRNA levels at 6 hours and a 5- to 9-fold increase still demonstrable at 48 hours (Fig 5). The tumor promoter PMA caused a sustained elevation of u-PAR mRNA initially detectable after 4 hours of stimulation with maximal 12.3 ± 2.5-fold elevation from 9 hours onward throughout the stimulation period of 48 hours (Fig 6).

Effect of DRB on mitogen-induced u-PAR mRNA elevation. In order to determine whether the mitogen-stimulated increase in u-PAR mRNA levels was due to increased u-PAR mRNA stability or resulted from enhanced transcriptional activity we included transcription inhibitors in our stimula-
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Fig 4. Time-dependent effect of thrombin on thrombin receptor mRNA levels in bovine vascular SMC. (A) Bovine vascular SMC were exposed to thrombin (40 U/ml) and the cells harvested for total RNA isolation at time point "0" (lane 1), 1 hour (lane 2), 2 hours (lane 3), 3 hours (lane 4), 4 hours (lane 5), and 5 hours (lane 6). Twenty micrograms of total RNA were hybridized overnight with \(10^7\) cpm of \(\text{[}\overset{32}{P}\text{]}\)u-PAR cRNA, \(10^6\) cpm of APP cRNA, and \(5 \times 10^5\) cpm of the thrombin receptor (TR) cRNA at 42°C. RNase protection assays performed as described under Materials and Methods. The protected fragments (u-PAR: 279 bp; APP: 229 bp; TR: 417 bp) were electrophoresed on TBE/polyacrylamide/urea-gels, dried and exposed to x-ray films for autoradiography. M: \(\text{[}\overset{32}{P}\text{]}\)-labeled Lambda HindIII Phi X Hae III DNA markers. (B) The signal intensity obtained by the u-PAR and TR probes was determined by laser densitometry, normalized to the signal from the internal standard APP and the relative amount of u-PAR and TR mRNA expressed as “fold induction” by defining the mRNA level at time point “0” as “1”.

DISCUSSION

Intimal hyperplasia is a prominent feature in the developing primary atheromatous plaque and in post-angioplasty restenosis. SMC have been demonstrated to be the main component in human atherosclerotic lesions. Proliferation and migration of SMC from the arterial media to the intima are essential in these processes requiring the transformation of a quiescent contractile SMC into a migratory and secretory SMC phenotype. Cell surface u-PA-mediated plasmin formation is thought to play an important role in cell invasion at least in part due to plasmin’s ability to digest extracellular matrix proteins and to activate procollagenase. The fine regulation of pericellular u-PA/plasmin-mediated proteolytic activity in tumor cells and transformed monocytoid U937 cells was shown to be under the control of several mitogens, which were shown to effectively modulate the expression of
The receptor for u-PA is another regulatory factor of cell surface plasmin generation. In fact, u-PAR numbers were directly correlated with cell mobility and invasiveness of tumor cells. We recently identified and characterized u-PAR on bovine aorta SMC. Receptor numbers and affinities were determined on the cell surface by radioligand binding experiments after stimulation of SMC by thrombin, three additional mitogens implicated in atherogenesis, bFGF, TGFβ, and PDGF, as well as the tumor promoter PMA. Functional plasminogen activation assays on the SMC surface demonstrated that plasmin generation was significantly increased by cell surface u-PAR-bound pro-u-PA than compared with experiments under fluid phase conditions. However, the effect of these mitogens on u-PAR mRNA levels in vascular SMC has not been systematically investigated.

Thrombin, a well recognized SMC mitogen, increased u-PAR mRNA levels 10 ± 2.3-fold in bovine vascular SMC revealing peak levels within 6 to 9 hours followed by a gradual decline to basal levels at 48 hours. The effect of thrombin on u-PAR mRNA requires intact catalytic activity of the protease. This observation is in agreement with a novel proteolytic mechanism by which thrombin activates the thrombin receptor described on platelets and endothelial cells. A 100 amino acid residues extracellular NH2-terminal domain of this thrombin receptor contains a putative thrombin cleavage site, L-D-P-WS. It was determined that the new NH2-terminal peptide region spanning 14 amino acids and arising from R-41/S-42 cleavage became a tethered ligand capable of activating the receptor. Synthetic peptides from 5 to 14 amino acids in length mimicking the new NH2-
TB-Elpolyacrylamide-gels, of R-41/S-42 and perhaps other sites within the thrombin desensitization may occur through the proteolytic cleavage of cells. However, the time-dependence of induction was different. Whereas TGFP, increased U-PAR mRNA levels in bovine aorta SMC. Bovine aorta SMC were treated with TRAP also produced a time-dependent rise in U-PAR mRNA expression with steady 5 to 10-fold elevated levels at 48 hours. The sustained elevation of u-PAR mRNA produced by PMA is consistent with data on u-PAR mRNA levels in PMA stimulated U937 cells. With respect to the prolonged effect of bFGF on u-PAR mRNA levels it might be that the complexity and diversity of the bFGF/bFGF receptor system could account for the extended effects observed on bFGF stimulation.

In order to determine whether the increase in u-PAR mRNA levels might be due to enhanced transcriptional activity or was a consequence of increased mRNA stability we included the specific RNA polymerase II inhibitor DRB into the mitogen stimulation experiments. The u-PAR message levels were followed up to 24 hours in the presence or absence of DRB by RNase protection assays. The case of TGFB1 stimulation we observed an approximate 3-fold reduction in u-PAR mRNA steady state levels within 3 hours after the addition of DRB to the cell culture supernatants compared with controls without DRB. These findings indi-

Fig 6. Time-dependent effect of PMA on u-PAR mRNA levels in bovine vascular SMC. Bovine aorta SMC were treated with 200 nmol/L PMA for 15 minutes (lane 2), 30 minutes (lane 3), 1 hour (lane 4), 2 hours (lane 5), 4 hours (lane 6), 6 hours (lane 7), 9 hours (lane 8), 12 hours (lane 9), 24 hours (lane 10), and 48 hours (lane 11) and subsequently total RNA isolated. Untreated SMC served as controls (lane 1). Five micrograms of total RNA were hybridized overnight with 2.5 x 105 cpm of [32P]-labeled u-PAR cRNA and 2 x 104 cpm of the internal standard APP at 42°C and RNase protection assays performed as described under Materials and Methods. The protected fragments were precipitated, resuspended, and electrophoresed on TBE/polyacrylamide/urea-gels, which were dripped and exposed to x-ray films for autoradiography. M: [32P]-labeled Lambda HindIII Phi X Hae III DNA markers.

terminus (thrombin receptor activation peptide or TRAP) were shown to activate the thrombin receptor in the absence of thrombin thus providing a solid argument for the tethered ligand theory. In our experiments, stimulation of SMC with TRAP also produced a time-dependent rise in u-PAR message similar to that seen on stimulation with catalytically active thrombin indicating the involvement of the thrombin receptor. Exposure of SMC to thrombin for 30 minutes was as effective as continuous thrombin stimulation indicating that a brief time period is sufficient to cleave and thus activate all cell surface thrombin receptor sites.

The increase in u-PAR mRNA levels in SMC was also under the control of PDGF and TGFB1, with 12 ± 2.5-fold and 5.5 ± 0.9-fold elevated mRNA levels, respectively. These observations confirm data of u-PAR mRNA upregulation (15-fold) in TGFB1-stimulated A549 lung carcinoma cells. However, the time-dependence of induction was different. Whereas TGFB1 increased u-PAR mRNA levels in SMC in a transient fashion no decline in the message levels was noticed on TGFB1-stimulated A549 tumor cells. The transient rise and subsequent decline in u-PAR message levels after the addition of DRB to the cell culture supernatants was similar to that provoked by PDGF and thrombin. With respect to thrombin a loss of responsiveness to the enzyme following an initial response has been described as a mechanism to modulate thrombin-mediated biological responses and to limit the duration of intracellular signalling. It has been suggested that desensitization of the thrombin receptor is involved or that receptor desensitization may occur through the proteolytic cleavage of R-41/S-42 and perhaps other sites within the thrombin receptor molecule, which would render the receptor unable to interact a second time with thrombin. Resensitization of cells to thrombin requires up to 24 hours a time frame very similar to that observed for u-PAR mRNA upregulation where the initial peak is observed within 6 to 9 hours followed by the return to basal levels at 48 hours. The biological responses of cells to TGFB1 and PDGF are also strongly dependent on interactions with corresponding cell surface receptors and attenuation of signal transduction might result from the down-regulation of receptors via ligand-induced internalization and subsequent lysosomal degradation or receptor phosphorylation.47,48

In contrast to the three mitogens described above, PMA and bFGF provoked a more prolonged effect on u-PAR mRNA expression with steady 5 to 10-fold elevated levels at 48 hours. The sustained elevation of u-PAR mRNA produced by PMA is consistent with data on u-PAR mRNA levels in PMA stimulated U937 cells. With respect to the prolonged effect of bFGF on u-PAR mRNA levels it might be that the complexity and diversity of the bFGF/bFGF receptor system could account for the extended effects observed on bFGF stimulation.

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Fig 7. Effect of the RNA-polymerase II inhibitor DRB on u-PAR mRNA levels induced by thrombin and other mitogens. Bovine vascular SMC were exposed to thrombin (A), bFGF (B), PMA (C), and TGFB1 (D) for 3 hours followed by the addition of DRB to the cell culture supernatants (E). At distinct time intervals cells were harvested, total RNA isolated, and RNase protection assays performed as described above. Controls served as mitogen-stimulated SMC in the absence of DRB (F). The relative abundance of u-PAR mRNA was determined by the signal intensity of the protected fragments by laser densitometry and the measurements normalized as outlined above. The values at each time point are given as "fold induction" by defining the message level at time point "0" as "1". Each value represents the average of duplicate experiments.
cate the necessity of ongoing transcription for the effect of TGFβ3, rather than prolongation of u-PAR message half life. However, a combination of both, increased transcriptional activity and mRNA stabilization cannot be excluded. These findings on bovine vascular SMC are consistent with data from TGFβ3-stimulated A 549 lung carcinoma cells. In this study the investigators suggested that the approximately 3-fold increase in the transcriptional activity of the u-PAR gene accounted at least in part for the TGFβ1 induced increase of u-PAR mRNA levels.40 In contrast, thrombin, bFGF and PDGF-stimulated increases in u-PAR message levels were not significantly altered in the presence of DRB, suggesting that the accumulation of u-PAR mRNA was mainly due to a message stabilization; the half life of u-PAR mRNA under unstimulated conditions was estimated under DRB treatment to range between 5 to 7 hours. As a further control for the specific mitogen effects on u-PAR mRNA levels we determined c-myb mRNA levels. c-myb RNA has been shown to be expressed at low levels in quiescent bovine SMC,27 but increases after stimulation of SMC 3 to 4-fold during late G1 and remained at this level during S-phase. These data indicated that the c-myb proto-oncogene is involved in cell cycle progression and SMC proliferation. We hybridized the c-myb probe with RNA from TGFβ1 or thrombin stimulated SMC in RNase protection assays. The slight increase in c-myb message levels under TGFβ1 stimulation did not change over the observed time span and did not decay under DRB. The c-myb RNA levels under thrombin stimulation were virtually unaltered when compared with unstimulated controls no matter if DRB was present or not, thus underlining the specific response of u-PAR mRNA levels on the different mitogen inductions.

Treatment of SMC with the protein synthesis inhibitor cycloheximide increased u-PAR mRNA levels in a time-dependent fashion confirming observations made on U93726 and A549 carcinoma cells.40 Stimulation by thrombin in combination with cycloheximide resulted in an additive effect but no superinduction was observed. These data suggest that protein synthesis is not required for the effect of thrombin on u-PAR mRNA levels. The decline following the initial rise in u-PAR mRNA levels observed on thrombin stimulation of SMC was abolished in the presence of cycloheximide. Translation is thought to play an important role in controlling mRNA degradation. Several explanations have been postulated. Cycloheximide could produce an increased stability of u-PAR mRNA by blocking de novo protein synthesis of labile or short-lived RNA degrading enzymes or negatively regulating proteins involved in the transcriptional control. However, the balance between negative and positive trans-acting factors implicated in gene transcription and the impact of RNA degrading enzymes on message abundance is complex and cannot be elucidated based on the data presented here.

Most of the mitogens are known to cooperate with each other or act as indirect mitogens through the endogenous induction of other growth factors. Thus, TGFβ1, PMA and thrombin were shown to induce the expression of PDGF.30,55 Indeed we observed additive effects on u-PAR mRNA expression on stimulation of bovine vascular SMC by thrombin in combination with each of the other mitogens. Moreover, elevated levels of u-PAR mRNA were still demonstrable at 48 hours, thus shifting the induction to a more prolonged effect in contrast to that observed on TGFβ1, thrombin, or PDGF alone.

It has been speculated that the effects of growth factors on the progression of atherosclerosis might be enhanced by the concomitant expression of corresponding receptors.50 In the present study on cultured bovine vascular SMC we demonstrated not only an increase in thrombin receptor mRNA on bFGF stimulation as previously demonstrated on rat vascular SMC,51 but also a significant accumulation of thrombin receptor mRNA on exposure to thrombin could be observed. Co-hybridization of total RNA from thrombin stimulated SMC with a thrombin receptor and a u-PAR cRNA probe revealed that the thrombin induced u-PAR mRNA increase was preceded by an approximately 2-fold transient increase in thrombin receptor message within about 60 minutes and a return to basal levels within 2 hours.

The in vivo relevance of the regulation of u-PAR expression on SMC by different mitogens can be deduced only with the elucidation of their presence and availability in the microenvironment of the atheromatous plaque. Thrombin is thought to be immobilized on extracellular matrix and thus could provide a potent stimulus for SMC activation.61 None of the other growth factors including bFGF, TGFβ1, or PDGF is expressed in normal arteries but can be induced in endothelial cells and SMC at the site of atherosclerotic lesions. In addition, bFGF, TGFβ1, and PDGF are available from macrophages and platelets that are contained within the atheromatous plaque.50,59 Thus, the increases in u-PAR mRNA levels by known SMC mitogens is complex and may require growth factor crosstalk and possible cascade-like mechanisms, which are currently under investigation.

ACKNOWLEDGMENT

The authors thank Drs H. Graeff, M. Schmitt, O. Wilhelm, V. Magdolen (Frauenklinik der Technischen Universität München), and Dr M.J. Atkinson (GSF München) for their support and helpful suggestions in the course of the experiments concerned with u-PAR mRNA stability and transcriptional activation.

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