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Activation of tissue plasminogen activator gene transcription by Neovastat, a multifunctional antiangiogenic agent☆

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Abstract

We recently reported that Neovastat, an antiangiogenic drug that is currently undergoing Phase III clinical trials for the treatment of non-small cell lung cancer, may inhibit angiogenesis through an increase in tPA activity. Here, we show that Neovastat also stimulates tPA gene transcription in endothelial cells, in a TNFα-like manner. RT-PCR analysis and gene reporter assays using the human tPA promoter indicated that upregulation of the tPA gene transcription by both Neovastat and TNFα was correlated with the phosphorylation of JNK1/2 and of IκB and that SP600125 and BAY11-7082, inhibitors of JNK and IκK, respectively, inhibit the increase of tPA gene transcription induced by Neovastat and TNFα. These results suggest that Neovastat induces tPA gene transcription through activation of the JNK and NFκB signaling pathways, leading to an increase of tPA secretion by endothelial cells. This may lead to the localized destruction of the fibrin provisional matrix that is necessary for neovessel formation and thus contribute to the reported antiangiogenic properties of this compound.

There is compelling evidence that tumor-induced angiogenesis represents a central process involved in the aggressive growth of tumors and of their metastases [1]. As such, the targeting of angiogenesis as a means of blocking tumor progression has driven considerable interest in recent years, leading to the identification of a variety of angiostatic proteins and molecules that specially inhibit endothelial cell proliferation, migration, and vessel formation (reviewed in [2]). In this respect, interference with cell adhesion and migration of endothelial cells (EC) is a common mechanism by which antiangiogenic molecules elicit their biological effects [3].

Proteolytic breakdown of the extracellular matrix (ECM) by matrix metalloproteinases (MMP) and plasmin has received widespread endorsement as an essential process underlying angiogenesis [4], enabling migration of EC and their morphogenic differentiation into tubular structures. Plasmin is a broad spectrum protease that mediates proteolysis of the ECM by degrading fibrin and several matrix molecules [5]. As such, activation of the zymogen plasminogen to plasmin by urokinase- and tissue-type plasminogen activators has been suggested to represent an important event in cell migration [6]. While this hypothesis has been strongly demonstrated for uPA-mediated plasmin generation [4,6], the role of tPA-mediated plasmin activation in angiogenesis is much less understood.

Endothelial cells synthesize, store, and release tPA into the circulation and, as such, are considered to be the predominant source for tPA in vivo [7]. The released tPA is a key enzyme in fibrinolysis due to its ability to significantly increase the cleavage of fibrin-bound plasminogen into plasmin, leading to fibrin degradation [8].

☆ *Abbreviations:* MMP, matrix metalloproteinase; tPA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

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The role of this tPA-mediated fibrinolytic activity in angiogenesis remains largely unknown [4] but is likely to be important given the crucial importance of the provisional fibrin provisional matrix for neovessel formation [9]. However, by contrast to uPA, for which considerable evidence indicates that upregulation of the enzyme correlates with the aggressiveness of tumors [10,11] several observations suggest that high tPA levels rather correlate with good prognosis of various tumors [12,13] whereas lower tPA levels have been associated with malignant tumors [14]. This suggests that increase in either tPA activity or expression levels may be beneficial, possibly due to the overstimulation of plasmin generation by tPA that may induce the degradation of the pro-angiogenic fibrin matrix, resulting in the inhibition of angiogenesis [15].

Neovastat is a naturally occurring inhibitor of angiogenesis derived from marine cartilage [16]. There is now considerable evidence that the clinical benefits observed upon Neovastat treatment rely on the presence of multiple angiogenesis inhibitors, including inhibitors of MMP activities [17] and of VEGF-mediated signaling events [18], as well as on the presence of an endothelial-specific pro-apoptotic activity [19]. Recently, we also reported that Neovastat specifically stimulates tPA-dependent plasmin generation through an increase in the affinity of the enzyme towards plasminogen [20].

In this work, we report that, in addition to its stimulatory effect on tPA activity, Neovastat also markedly stimulates tPA expression in endothelial cells through an increase in the transcription of the tPA gene. In addition, we show that this transcriptional activation is associated with the TNFα-like activation of both JNK and NFκB signaling pathways. These results thus support the concept that modulation of tPA activity both at the enzymatic and transcriptional levels may represent an important feature responsible for the antiangiogenic activity of Neovastat.

Materials and methods

Reagents. Neovastat (Æ-941) was obtained from Æterna Laboratories (Québec City, QC, Canada. Dulbecco's modified Eagle's media (DMEM) low glucose, antibiotic mixture (penicillin, streptomycin), and glutamine were purchased from Gibco (Grand Island, NY, USA). Trypsin-EDTA for endothelial cells, ethidium bromide, *n*-butyric acid, and actinomycin D were obtained from Sigma (St. Louis, MO, USA). b-FGF was obtained from Upstate (Lake Placid, NY, USA). TNFα, human recombinant tPA, and uPA (hr-tPA and hr-uPA) were obtained from Calbiochem (San Diego, California, USA). Human plasminogen and the Fugene 6 transfection reagent were purchased from Roche Diagnostics (Laval, QC, Canada). Antibodies against phospho-JNK1/2, JNK1/2, and phospho c-Jun were obtained from Cell Signaling Technology (Pickering, ON, Canada). Mouse and rabbit HRP-conjugated antibodies were purchased from Jackson ImmunoResearch (Mississauga, ON, Canada). TRIzol Reagent, superscript one-step RT-PCR with Platinium *Taq* were from Invitrogen (Burlington, ON, Canada). PVDF transfer membranes and Western Lightning Chemiluminescence Reagent Plus were obtained from Perkin–Elmer Life Sciences (Boston, MA, USA). Cycloheximide, SP600125, and BAY11-7082 were obtained from Biomol Research Labs (Plymouth Meeting, PA, USA). Basic pGL3 and the luciferase assay reagents were purchased from Promega (Madison, WI, USA). pSEAP was obtained from Clontech (Palo Alto, California, USA). MicroBCA reagents were purchased from Pierce (Rockford, IL, USA).

Cell Culture. Bovine aortic endothelial cells (BAEC) purchased from Clonetics and were cultured at 37 °C in DMEM low glucose, pH 7.3, supplemented with 10% fetal bovine serum (FBS), containing 10 ng/ml b-FGF, and 100 U/ml penicillin/streptomycin/glutamine. BAEC were used up to passage 12.

Preparation of cell lysates. BAEC were cultured in six-well plates until 90% confluency and treated with several drugs in DMEM containing 0.5% FBS. Cells were washed with phosphate-buffered saline (PBS), pH 7.4, and then scrapped in lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, and 0.2 mM PMSF). Cells were lysed for 30 min on ice, with occasional vortexing, and cell debris were removed by centrifugation at 10,000*g*, for 10 min at 4 °C. Proteins contained in cell lysates were quantitated using the MicroBCA method.

Casein–plasminogen zymography. Twenty microliters of conditioned medium was resuspended in non-reducing sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.00625% bromophenol blue) and submitted to 10% polyacrylamide SDS–PAGE, using gels containing 1 mg/ml casein and 10 μg/ml plasminogen. Gels were then washed twice for 30 min in 2.5% Triton X-100 and rinsed with nanopure water. Gels were incubated at 37 °C in zymography buffer (100 mM Tris–HCl, pH 8.0, 50 mM EDTA) for 3 h. Digested areas were visualized by coloration of the gels with Coomassie blue.

RNA isolation. BAEC were cultured in six-well plates until 90% confluency and treated with drugs in DMEM containing 0.5% FBS. Total RNA was isolated from the treated cells using the TRIzol reagent, according to the instructions of the manufacturer. RNA was dissolved in 20 μl of DEPC-H_2O and quantitated at 260 nm.

Reverse transcription and polymerase chain reaction. One microgram of total RNA isolated from cells was amplified with superscript one-step RT-PCR with Platinium *Taq*, using specific primers designed for bovine tPA: sense, 5′-CTG CAC CTG AAA TCA GAC TCG C-3′ and antisense, 5′-CTG ATG ATG CCG ACC AAG GTC-3′; or for actin as a control. Reverse transcription and polymerase chain reaction (RT-PCR) was carried out in the following conditions: 1 cycle (50 °C, 30 min; 92 °C, 2 min) for the reverse transcription, 30 cycles (92 °C, 30 s; 50 °C, 30 s; and 72 °C, 45 s) for the amplification, and 1 cycle (72 °C, 7 min) for the final extension. Products were analyzed using 1.5% agarose gel containing ethidium bromide.

Western blotting. Twenty micrograms of proteins contained in cell lysates was resuspended in reducing sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.00625% bromophenol blue) and heated for 5 min at 100 °C. Samples were then separated by 10% SDS–PAGE under reducing conditions and transferred onto PVDF membranes. Membranes were saturated with 5% milk in TBS/0.1% Tween for 1 h at room temperature, incubated overnight at 4 °C with the indicated antibody (1/1000), and then incubated with the corresponding HRP-conjugated secondary antibody (1/10,000) for 1 h at room temperature. Immunocomplexes were visualized by the Western Lightning Chemiluminescence Reagent using Fuji Films.

Gene reporter assays. The human tPA promoter was cloned by PCR using HUVEC genomic DNA as the template, essentially as described [21]. Primers used were: sense, 5′-CGATCGGTACC TTTCGGGATGATTCAAGAGGATTAC-3′; antisense, 5′-CGATC AGATCTGAAAGAAGAGGAGACAGACCCCAAG-3′, where the underlined bases represent *Kpn*I and *Bgl*II restriction sites, respectively. PCR conditions used were (40 cycles): annealing at 52 °C for 1 min, elongation at 68 °C for 4 min, and denaturation at 94 °C for

1 min. The resulting PCR product (3.6 kb) was digested with *Kpn*I and *Bgl*II and cloned into the pGL3 luciferase reporter vector.

Transcient transfection of the reporter construct was performed using the Fugene 6 transfection reagent. BAEC were cultured in six-well plates until 60% confluency and transfected with a 50:1 ratio of a mixture containing the pGL3-tPApromoter-luciferase vector and pSEAP, encoding a constitutively secreted form of alkaline phosphatase. Cells were then incubated in medium containing 10% serum for 24 h and treated with the drugs for different times. Cells were lysed with passive lysis buffer (Promega) and luciferase activity was measured according to the manufacturer's instructions. Transfection efficiencies were normalized by measuring alkaline phosphatase activity in the conditioned media, using *p*-nitrophenylphosphate (*p*NPP) as a substrate.

The mercury pathway profiling system, in which a secretory alkaline phosphatase form is fused to promoters activated by different responsive elements, was used to monitor the effect of Neovastat on different response elements. Cells were transfected with the various constructs and aliquots of the conditioned media were collected at different times. SEAP activity was measured by the hydrolysis of pNPP, as described by the manufacturer (Clontech).

Results

Neovastat induces secretion of tPA by endothelial cells that correlates with increased tPA mRNA levels

It is well established that endothelial cells are the most important source of tPA in vivo [7]. In order to investigate the effect of Neovastat on tPA expression by EC, BAEC were treated with the compound and the presence of tPA in the conditioned medium was monitored by zymography. tPA is secreted as a 70 kDa precursor protein (the one-chain form) that can be cleaved by plasmin to generate a 35 kDa form (two-chain form) [22,23], which has been proposed to be more active than the 70 kDa precursor form. As shown in Fig. 1A, addition of Neovastat to BAEC induces a concentration-dependent secretion of both forms of tPA. Secretion of tPA form could be detected with doses of Neovastat as low as 5 μg/ml and reached a maximum at 100 μg/ml. At this dose of Neovastat, the two-chain form is detectable as early as 30 min after the addition of the compound. These results indicate that Neovastat markedly increases the secretion of tPA by endothelial cells.

In order to determine if the observed increase in tPA expression was related to an effect of Neovastat on tPA mRNA levels, endothelial cells were treated with Neovastat and the levels of tPA mRNA were monitored by RT-PCR. As shown in Fig. 1, Neovastat induced a marked increase in tPA mRNA, in a dose-dependent manner (Fig. 1B). Time-course analysis indicates that tPA mRNA levels are enhanced as early as 30 min of treatment with Neovastat (Fig. 1B). We next compared the extent of stimulation of tPA mRNA levels by Neovastat to those achieved with known inducers of this gene. For example, it has been shown that TNFα induces tPA release by endothelial cells in vivo in humans

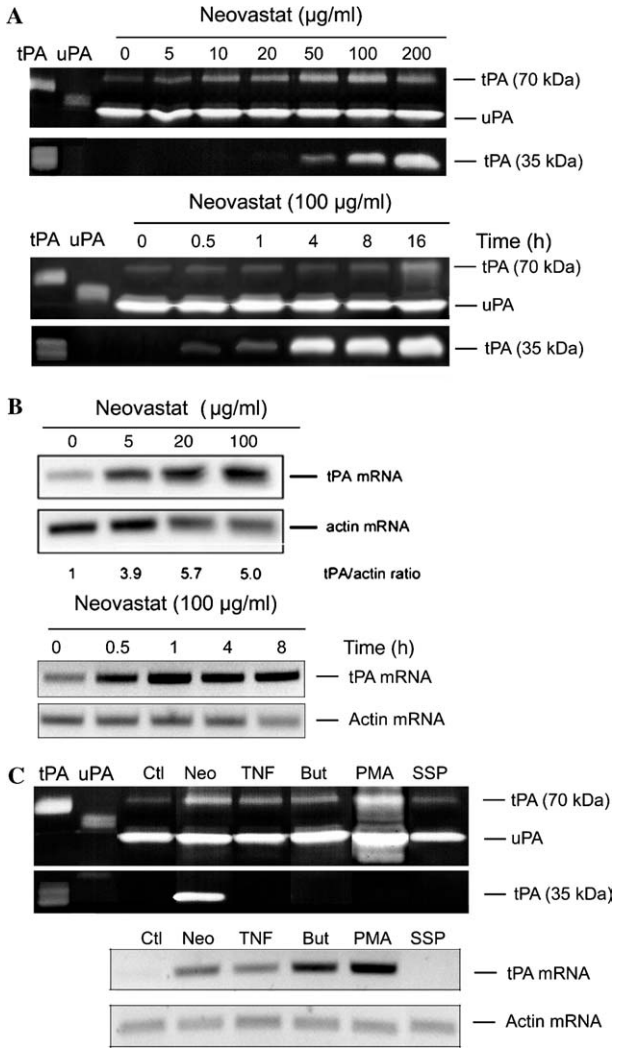

Fig. 1. Induction of tPA secretion and mRNA levels by Neovastat in endothelial cells. (A) Neovastat induces secretion of tPA. Confluent BAEC were treated overnight with the indicated concentrations of Neovastat or with 100 μg/ml Neovastat for the indicated periods of time and secreted tPA was monitored by casein–plasminogen zymography. Human recombinant tPA and uPA (hr-tPA and hr-uPA) were used as molecular weight markers to identify single-chain and two-chain tPA as well as uPA activities. (B) Dose-dependent upregulation of tPA mRNA levels by Neovastat. Confluent BAEC were treated for 4 h with increasing concentrations of Neovastat or with 100 μg/ml Neovastat for different periods of time. Total RNA was isolated from cells and subjected to RT-PCR, using specific primers designed to amplify tPA and actin mRNAs. (C) Comparative analysis of tPA induction by Neovastat, TNFα, sodium butyrate, PMA, and staurosporine (SSP) in endothelial cells. Confluent BAEC were treated with 100 μg/ml Neovastat, 50 ng/ml TNFα, 300 μg/ml sodium butyrate, 20 ng/ml PMA or 200 nM staurosporine for 4 h, secreted tPA was monitored by casein–plasminogen zymography, and tPA mRNA levels were analyzed by RT-PCR.

[24] and increases tPA mRNA levels in human pulp and gingival fibroblasts in vitro [25]. It is also well established that the short-chain fatty acid sodium butyrate [26,27] and PMA [28] also stimulate tPA gene expression in endothelial cells. As shown in Fig. 1C, all these agents

induce secretion of the 70 kDa form of tPA in conditioned media and upregulate tPA mRNA to an extent similar to that observed with Neovastat (Fig. 1C). Interestingly, Neovastat was however the only compound that promoted the release of the 35 kDa form of tPA. These effects of Neovastat and TNF were not a consequence of EC apoptosis triggered by both compounds [19] since treatment of BAEC with staurosporine, a well-described apoptosis inducer, had no effect on both tPA secretion and mRNA upregulation (Fig. 1C).

Neovastat induces the SAPK/JNK pathway

The increase of tPA mRNA levels by Neovastat suggests that the compound may elicit this effect through activation of intracellular signaling pathways. In this respect, very few reports have documented the activation of signaling pathways by treatment of EC with antiangiogenic agents. Angiostatin induces focal adhesion kinase activity, possibly leading to focal adhesion breakdown [29] while endostatin increases tyrosine kinase signaling [30], and both events have been suggested to be important for the induction of EC apoptosis by these proteins [29,30]. However, besides angiostatin, which has been shown to upregulate E-selectin levels in BAEC [31], the influence of these events on gene transcription remains largely unknown.

The similarities between Neovastat and TNFα led us to investigate whether the increase in tPA mRNA levels by Neovastat involves signaling pathways similar to those activated by TNFα. In this respect, Jun amino-terminal kinases (JNK1/2/3) also called stress activated proteins kinase (SAPK) [32], and NFκB [33] are two key pathways that are known to be stimulated by cytokines like TNFα and the effect of Neovastat on the activation of these pathways was thus investigated. As shown in Fig. 2, addition of Neovastat to BAEC promotes a marked increase in the activation of the SAPK/JNK signaling pathway in endothelial cells, as reflected by the increased phosphorylation of JNK1 and JNK2. The kinetics of JNK1/2 phosphorylation by Neovastat was slower than that achieved by TNFα, with maximal phosphorylation by Neovastat observed at 1 h compared to 5 min for TNFα. This activation of JNK by both Neovastat and TNFα was correlated with the phosphorylation of the transcription factor c-Jun, suggesting that this nuclear factor is likely to influence the regulation of tPA expression.

IκB is the prototypical NFκB inhibitor protein. Upon stimulation, it is phosphorylated on Ser-32 and Ser-36 and targeted to ubiquitin-dependent degradation allowing the shuttling of the transcription factor to the nucleus [33]. We thus used Western blot to assess the abundance of IκB following stimulation of the cells with Neovastat and TNFα. As shown in Fig. 2B, Neovastat induced a strong increase in the phosphorylation of IκB

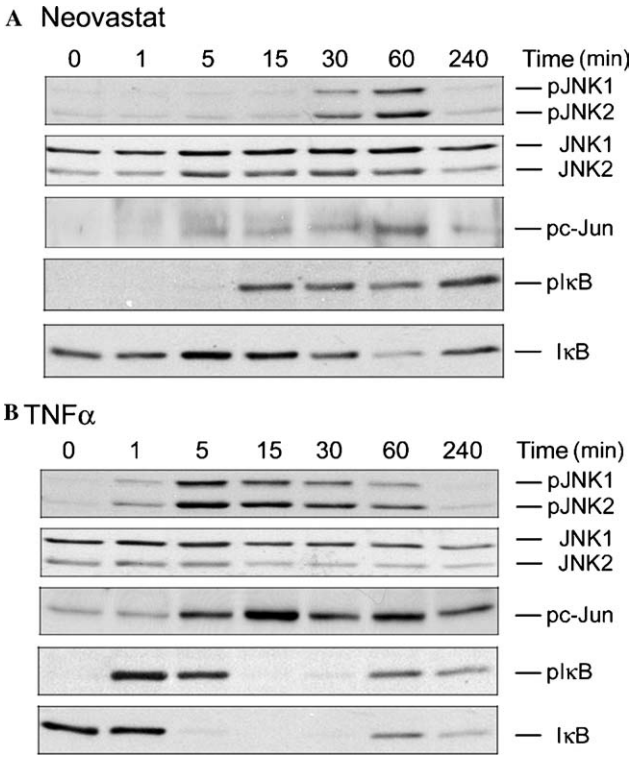

Fig. 2. Effect of Neovastat and TNFα on the SAPK/JNK and NFκB pathways. Confluent BAEC were treated with 100 μg/ml Neovastat (A) or with 50 ng/ml TNFα (B) for the indicated periods of time. Cell lysates were prepared and immunoblotted using phospho JNK1/2, phospho c-jun, and phospho IκB. The blots were then reprobed for total JNK1/2 and IκB.

that correlates with the degradation of this regulatory subunit of the transcription factor. This effect was here again similar to that achieved following treatment of the cells with TNFα, thus confirming the similarity between Neovastat and TNFα.

Inhibition of JNK and NFκB pathways blocks tPA mRNA upregulation by Neovastat

We next determined if the activation of these two important pathways by Neovastat could participate in the induction of tPA mRNA. As expected, upregulation of the mRNA levels induced by Neovastat and TNFα was completely inhibited by actinomycin D, an inhibitor of transcription, suggesting that the observed increase is not due to mRNA stabilization but rather to induction of tPA gene transcription (Fig. 3A). A relationship between JNK and NFκB activation and tPA mRNA upregulation induced by Neovastat was investigated using SP600125, a JNK inhibitor that inhibits JNK and thus c-Jun phosphorylation [34] and BAY11-7082, a IκK inhibitor [35]. As shown in Fig. 3A, pretreatment of the cells with both inhibitors prior to incubation with Neovastat resulted in a marked inhibition of tPA mRNA induction by the compound, suggesting that

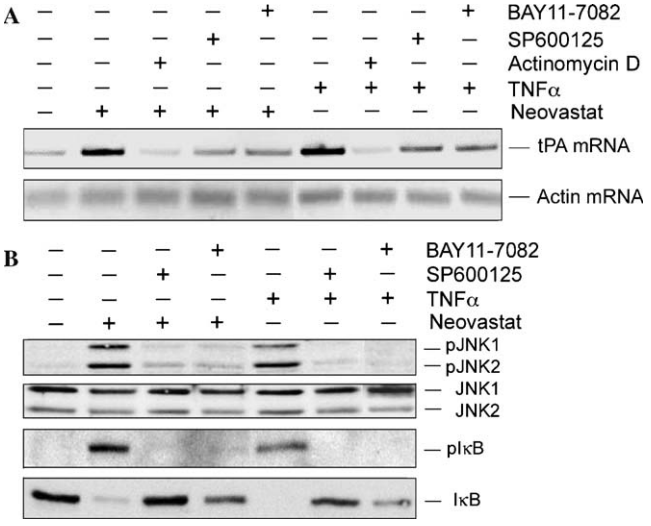

Fig. 3. tPA gene induction by Neovastat is mediated by the SAPK/JNK and NFκB pathways. (A) RT-PCR analysis of tPA mRNA levels. Confluent BAEC were pre-incubated with 10 μg/ml actinomycin D, 10 μM SP600125 or 10 μM BAY11-7082 for 30 min at 37 °C and then incubated in the absence or in the presence of 100 μg/ml Neovastat or 50 ng/ml TNFα for 4 h. Actin and tPA mRNAs were reverse-transcribed as described previously. (B) Immunoblot analysis of treated cells. Cells were pre-incubated as described in (A) and incubated for 1 h in the absence or in the presence of 100 μg/ml Neovastat or 50 ng/ml TNFα. Cell lysates were immunoblotted with phospho JNK1/2 and phospho IκB. The blots were then reprobed for total JNK1/2 IκB.

both pathways play an important role in the induction of tPA mRNA by Neovastat. Under these conditions, Neovastat-mediated JNK phosphorylation was abolished by treatment with SP600125, confirming that the compound inhibits the activation of the enzyme. In a similar manner, BAY11-7082 completely inhibits the phosphorylation of IκB. Interestingly, inhibition of JNK abolished activation of the IκB pathway and vice versa, suggesting that the two pathways are closely linked. However, SP600125 has recently been reported to inhibit a number of kinases in addition to JNK [36] and we cannot rule out the participation of other pathways in the observed effect. However, it is unlikely that the inhibition of IκB phosphorylation is due to a non-specific effect of the compound on IκK since previous work has shown that it does not inhibit this enzyme [37]. Overall, these results indicate that Neovastat induces activation of the JNK and NFκB pathways and that these events are likely to play an important role in the upregulation of tPA mRNA levels by the drug.

Neovastat activates the human tPA promoter

The effect of Neovastat on tPA gene transcription was next evaluated using a 3.1-kilobase pair human tPA promoter linked to a luciferase reporter (ptPA.Luc). BAEC were transfected with the construct, treated with Neovastat or TNFα and luciferase activity was monitored in cell lysates. As shown in Fig. 4A, Neovastat-induced activation of the tPA promoter was maximal at 4 h while the peak of activation by TNFα occurred as early as 15 min. In both cases, luciferase activity in the cell lysates markedly declined afterwards, possibly reflecting EC apoptosis induced by both agents [19]. Neovastat-induced activation of the tPA promoter was dose-dependent, a half-maximal stimulation being observed at 30 μg/ml (Fig. 4B). The contribution of the JNK and NFκ B pathways to tPA gene transcription was next evaluated. As shown in Fig. 4C, both SP600125 and BAY11-7082 strongly inhibited luciferase activity induced by Neovastat and TNFα, thus reinforcing the concept that induction of the tPA gene by Neovastat occurs via activation of these pathways.

We further investigated the transcriptional activation induced by Neovastat using plasmids containing different response elements fused to a secreted form of alkaline phosphatase (SEAP). In this gene reporter assay, the activity of the response elements can be easily monitored by measuring SEAP activity in the culture medium. Under these conditions, we observed that addition of Neovastat induced significant activation of the serum (SRE), NFκB, and AP-1 response elements, whereas it had no effect on a variety of other elements (Fig. 4D). Since the induction of the AP-1 and SRE response elements can occur by activation of the JNK pathway whereas that of the κB element is triggered by the activation of the NFκB signaling pathway, these results provide further support that the activation of these pathways by Neovastat results in gene transcription. Moreover, given the variety of genes that are controlled by these elements, these results suggest that, in addition to the tPA gene, Neovastat is likely to induce transcriptional activation of other genes.

Discussion

Tissue-type plasminogen activator specifically converts circulating plasminogen to the active proteinase plasmin, a key trypsin-like protease that degrades the extracellular matrix and plays an essential role in various processes including clot dissolution, tissue remodeling, invasive growth of cancer cells, and angiogenesis [4]. In this latter case, there is considerable evidence that the net balance between molecules that have positive and negative regulatory activities is a major determinant in controlling neovessel formation. In particular, balanced proteolytic degradation is essential for angiogenesis since excessive proteolysis of the matrix scaffold results in loss of adherence and inhibition of invasion and vascular structure formation [38].

In this respect, there is recent evidence that increased tPA activity could interfere with angiogenesis. Insoluble endostatin, which consists of amyloid fibers, markedly increases tPA activity [39,40], resulting in endothelial

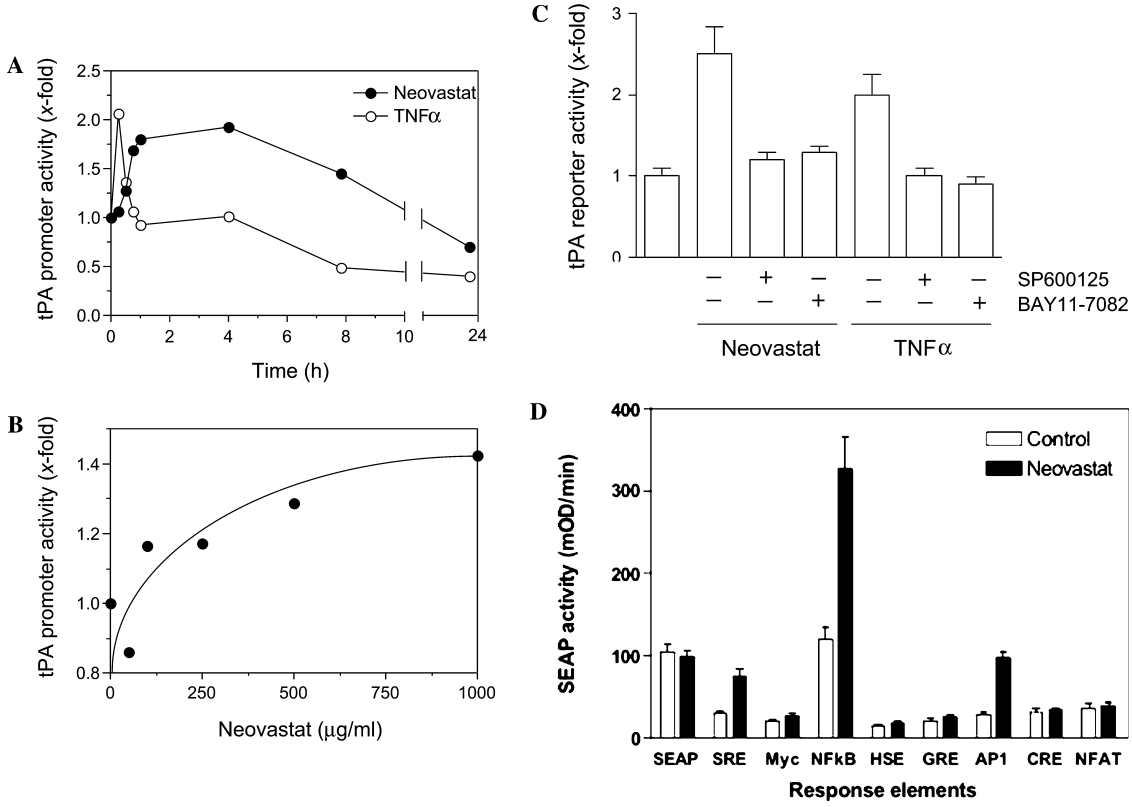

Fig. 4. Neovastat activates the tPA promoter through the SAPK/JNK and NFκB pathways. Neovastat activates tPA promoter in a time- and dose-dependent manner. Confluent BAEC co-transfected with pGL3-tPApromoter-luc and pSEAP were treated with 50 ng/ml TNFα or 500 μg/ml Neovastat for the indicated periods of time (A) or for 4 h with increasing concentrations of Neovastat (B). Luciferase activity was measured in cell extracts by luminescence. Transfection efficiency was normalized by measuring secreted alkaline phosphatase activity in the cell media. (C) Confluent BAEC co-transfected with pGL3-tPApromoter-luc and pSEAP were pre-incubated with 10 μM SP600125 or 10 μM BAY11-7082 for 30 min at 37 °C and treated with 500 μg/ml Neovastat for 4 h or with 50 ng/ml TNF-α for 15 min. Luciferase activity was measured in cell extracts by luminescence. (D) Cells were transfected with plasmids containing the indicated response elements and treated with 100 μg/ml for 8 h with Neovastat. Aliquots of the resulting conditioned medium were used to measure alkaline phosphatase activity, using pNPP as the substrate. SEAP indicates a control vector in which SEAP is constitutively secreted by the cells.

cell detachment [40]. Similar results were recently obtained using Neovastat, which markedly increases the catalytic efficiency of tPA through an increase of the affinity of the enzyme for plasminogen [20]. These observations are in agreement with reports showing that high tPA content and activity correlates with good prognosis in melanoma and breast cancer patients and similar correlations were also reported for other types of tumors ([11,12], and references therein). In addition, mice injected with colon cancer cells overexpressing tPA had a lower number of liver metastases and higher survival rate than those injected with untransfected cells [41], further suggesting that tPA has a positive role in cancer. Human plasma treated with tPA in the presence of a reducing agent (captopril) was recently shown to possess significant antiangiogenic activity [42], again suggesting that increased tPA activity could be a major mechanism by which antiangiogenic molecules inhibit angiogenesis.

In this study, we report for the first time that induction of the tPA gene, resulting in increased secretion of the protein by endothelial cells, could represent another mechanism by which an antiangiogenic agent can influence the fibrinolytic system and thus contribute to its antiangiogenic properties. Interestingly, we also observed that the treatment of the cells with Neovastat resulted in the production of the two-chain form of tPA. Single-chain tPA has limited plasmin-producing activity unless the cofactor fibrin is present in contrast to the two-chain tPA which exhibits full activity without fibrin [43]. The conversion of single-chain tPA to two-chain tPA by Neovastat could thus significantly contribute to the stimulatory effect of this compound on fibrinolysis.

The induction of tPA gene transcription by Neovastat was relatively rapid and most likely involved activation of at least two key signaling pathways that lead to gene transcription, JNK and NFκB. The stimulatory effect of Neovastat on these pathways occurred to an extent similar to that achieved by TNFα, a prototypical activator of both pathways, although it was kinetically slower. Activation of both pathways was transient, in a manner typical for early steps in the signaling cascade,

and are likely involved in the resulting increase in tPA gene transcription since pharmacological inhibitors of both pathways suppress this gene induction. This raises the interesting possibility that Neovastat may use pathways similar to those triggered by TNFα in order to increase tPA gene transcription. Interestingly, the stimulation of these two pathways by Neovastat is likely to influence the transcription of several genes since response elements under the control of the JNK and NFκB pathways were found to be markedly activated by the compound.

At least one antiangiogenic protein, TSP-1, was shown to induce the activation of stress-activated protein kinases such as p38 [44] and JNK1 [45]. Interestingly, both pathways appear essential for the apoptosis-dependent inhibition of angiogenesis by this molecule [44,45], raising the possibility that the observed induction of JNK activation by Neovastat may play a role in the recently described induction of EC apoptosis by Neovastat [19]. Such a close relationship between JNK and apoptosis has been described in various cell types [46] and, recently, JNK was found to be essential for TNFα-induced apoptosis [47].

Although activation of the NFκB pathway by Neovastat was unexpected given the proposed role of this pathway in cell survival, there is increasing evidence that not all signals that activate NFκB are antiapoptotic and that, under specific circumstances, NFκB activation can render cells more sensitive to certain proapoptotic stimuli [48] by inducing the activation of proapoptotic gene such as Fas [49] or FasL [50]. Since Neovastat induces EC apoptosis, it is possible that the concomitant stimulation of both JNK and NFκB pathways by this compound results in such an increase and bypass, the survival signals triggered by NFκB.

In summary, we show that Neovastat induces an increase in the transcription of the tPA gene and that this effect is likely to involve activation of the JNK and NFκB pathways. Given the proposed role of these pathways as well as tPA-mediated fibrinolysis in apoptosis, these results suggest that increased tPA synthesis may represent a novel pathway by which antiangiogenic agents interfere with neovascularization. Further studies aimed at the characterization of the increased transcriptional activation of tPA as well as those of other genes by Neovastat should yield interesting new information on the mechanisms responsible for the antiangiogenic activity of this compound.

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