Stimulation of Tissue-Type Plasminogen Activator Expression by Retinoic Acid in Human Endothelial Cells Requires Retinoic Acid Receptor β2 Induction

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We previously showed the involvement of retinoic acid receptor α (RARα) in the induction of tissue-type plasminogen activator (t-PA) synthesis by RA in human umbilical vein endothelial cells (HUVECs). However, the rather slow onset of this induction of t-PA synthesis suggested an indirect role of RARα. Here, we show that the protein synthesis inhibitor, cycloheximide completely blocks the induction of t-PA by RA, which points to the need of an intermediary protein in t-PA stimulation. This intermediary protein is likely to be RARβ2 on the basis of the following findings: (1) the induction of RARβ by RA exactly precedes that of t-PA; (2) HUVECs with elevated RARβ mRNA levels show an undelayed t-PA induction on stimulation with RA, and this response can be almost completely inhibited with an RAR antagonist; and (3) an antisense oligodeoxynucleotide against the translation initiation site of RARβ mRNA greatly reduces the t-PA induction by RA. Thus, induction of t-PA by RA in HUVECs involves a 2-step mechanism requiring induction of RARβ via RARα, followed by induction of t-PA synthesis via RARβ. Each of these steps is shown to have a different activation profile with RA and 9-cis RA.

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SEVERAL CLINICAL STUDIES have shown an inverse correlation between blood fibrinolytic activity and the risk of thromboembolic disease. Consequently, much attention is being paid to the factors that govern blood fibrinolytic activity. Tissue-type plasminogen activator (t-PA), a key enzyme in the initiation of the fibrinolytic process, converts the zymogen plasminogen into the active enzyme plasmin. Plasmin can degrade fibrin, the matrix structure of a blood clot, into soluble fibrin degradation products. The importance of the role of t-PA in plasma fibrinolysis has recently been emphasized once more by Carmeliet et al, who showed that t-PA-deficient mice suffered an impaired thrombolytic potential in combination with an increased susceptibility towards endotoxin-induced thrombosis. The vascular endothelium plays an important role in determining plasma t-PA activity by synthesizing both t-PA and a specific inhibitor, plasminogen activator inhibitor-1 (PAI-1). We and others have previously shown that retinoids, ie, vitamin A and derivatives, rather specifically stimulate t-PA synthesis in cultured human endothelial cells, without markedly influencing PAI-1 synthesis. In vivo studies in rats also show that t-PA levels and retinoid status are correlated. Because of their physiological relevance and their potential as a pro-fibrinolytic drug, we are interested in the mechanism(s) by which retinoids stimulate t-PA expression in human endothelial cells.

Many of the biological effects of retinoids are assumed to be mediated via nuclear retinoid receptors. Two families of these receptors have been identified, the retinoic acid receptors (RARs) and the retinoid-X receptors (RXRs). Both RARs and RXRs are ligand-inducible transcription factors that modify the expression of specific genes by binding to specific DNA sequences, designated retinoid acid response elements (RAREs; for reviews, see Chambon and Giuguère). Recently, Bulens et al showed the presence of an RARE at position −7.3 kb in the t-PA promoter; cotransfection experiments in HT1080 cells showed that this RARE is functional. Both RARs and RXRs consist of three subtypes, designated α, β, and γ, and, because of alternative splicing and the use of different promoters, there are at least two different isoforms of each receptor subtype. Because RARs and RXRs function as either homodimers or heterodimers in binding to the RAREs, a great variety of homodimeric and heterodimeric combinations is possible. Each RAR/RXR subtype combination may specifically control the expression of a subset of RA target genes.

The question arises whether the induction of t-PA by retinoids in human endothelial cells is also mediated via RARs/RXRs and, if so, which receptor subtype(s) is involved. In a previous report, we showed by Northern analysis that cultured human umbilical vein endothelial cells (HUVECs) express all three RAR subtypes, RARα and RXRβ, albeit to a widely different extent. Using subtype-specific ligands and an antagonist with a high preference for RARα, we could identify RARα to be involved in the induction of t-PA by retinoids in HUVECs. However, the rather slow onset of this induction left open the possibility that the role of RARα in t-PA expression is an indirect one. We now show that the induction of t-PA by RA in HUVECs is dependent on ongoing protein synthesis, suggesting that, besides RARα, a second (transcription) factor is required that is not present or is present at levels that are too low under basal conditions. By applying antisense technology, we showed that this factor is likely to be RARβ2. Our data are consistent with a 2-step mechanism in which RARα mediates the induction of RARβ2, which subsequently mediates the induction of t-PA. On further analysis, each of these steps turned out to have its own ligand-dependency characteristics for RA, which preferentially binds to RARs, and for 9-cis RA, which is a ligand both for RARs and RXRs.

MATERIALS AND METHODS

Materials. All-trans RA, cycloheximide (CHX), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St Louis, MO).
9-cis RA and the RARs antagonist, Ro 41-5253, were kindly provided by Drs M. Klaus and C. Apfel (Hoffmann-LaRoche, Basel, Switzerland). Although Ro 41-5253 has a high preference for RARs, a large excess of Ro 41-5253 can also considerably reduce RARβ activation. Stock solutions of RA (10 mmol/L), 9-cis RA (10 mmol/L), Ro 41-5253 (10 mmol/L), and CHX were prepared in DMSO and stored at -20°C. Stock solutions were diluted with incubation medium to the final test concentrations immediately before the start of an experiment. All experiments involving retinoids were performed in subdued light, and the tubes containing the retinoid solutions were covered with aluminium foil. Sterile, pyrogen-free human serum albumin (20% wt/vol) was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Enzyme immunoassay kits for determination of human t-PA antigen (Thrombonostika t-PA) and PAI-1 antigen (“Immlyse”) were obtained from Organon Teknika (Boxtel, The Netherlands) and Biopool (Umeå, Sweden), respectively. Deoxyctydine 5’-[α-32P] triphosphate was from Amersham (Buckinghamshire, UK). Other materials used have been specified in the methods described or in the relating references.

Cell culture experiments. Endothelial cells were isolated from human umbilical cord veins using the method of Jaffe et al.16 The cells were cultured in fibronectin-coated dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20 mmol/L HEPES (pH, 7.4), 10% (vol/vol) heat-inactivated newborn calf serum, 10% (vol/vol) human serum, 150 μg/mL endothelial cell growth supplement,2 2 mmol/L L-glutamine, 5 IU/mL heparin, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO2 atmosphere. The medium was replaced every 2 to 3 days. Subcultures were obtained by trypsin/EDTA treatment of confluent monolayers at a split ratio of 1:3. The cells were used for experiments at the first or second passage. Sixteen to 24 hours before the start of an experiment, the media were replaced with incubation medium (DMEM supplemented with 20 mmol/L HEPES [pH, 7.4], 10% [vol/vol] human serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin). Conditioned media were obtained by incubating cells at 37°C with incubation medium containing the appropriate concentration of the test compound or stock solvent DMSO (final concentration, maximally 0.1% [vol/vol]). The conditioned media were centrifuged for 5 minutes at 8,000 rpm in a Microfuge centrifuge to remove cells and cellular debris. Conditioned media were stored at -20°C until use. The cells were washed with ice-cold phosphate buffered saline (0.15 mol/L NaCl/10 mmol/L Na2HPO4/1.5 mmol/L KH2PO4 [pH, 7.4]) and were used for isolation of RNA.

Antisense experiments. Ten hours before the start of an experiment, first-passage HUVECs were washed twice with DMEM and then incubated with DMEM supplemented with 20 mmol/L HEPES (pH, 7.4), 0.1% (wt/vol) human serum albumin, 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Subsequently, after a preincubation for 1 hour with 30 μmol/L antisense RARβ2 (Fig 1B) or control (random or anti RARβ4) oligodeoxynucleotides (oligo dNs; Fig 1B), the cells were stimulated with RA (final concentration, 1 μmol/L) spiked into the medium; the amount of t-PA antigen produced during 12 hours was measured. To ensure the presence of a sufficient amount of antisense oligo dNs during the experiment, a second dose of oligo dNs (30 μmol/L) was added 4 hours after the addition of RA. Antisense oligo dNs were purchased from Isogen Bioscience (Amsterdam, The Netherlands) and were high-performance liquid chromatography-purified.

Northern blot analysis. Total RNA from HUVECs (75 cm2) was isolated by the isothiocyanate/phenol/acid extraction method of Chomczynski et al.18 The RNA was dissolved in H2O, and the RNA concentration was determined spectrophotometrically. Equal amounts (10 to 15 μg) of RNA were separated on a formaldehyde/agarose gel19 and subsequently were capillary transferred to a Hybond N membrane according to the instructions of the manufacturer (Amersham). Hybridization was performed in 7% (wt/vol) sodium dodecyl sulfate (SDS), 1 mmol/L EDTA, 0.5 mol/L Na2HPO4/NaH2PO4 buffer (pH, 7.2) overnight at 63°C with 25 ng of probe labeled with the random primer method (Megaprime kit; Amersham). The membranes were subsequently washed twice with 2X SSC/1% (wt/vol) SDS (1× SSC: 0.15 mol/L NaCl and 0.015 mol/L Na2HPO4, buffer (pH, 7.4)) and then incubated with 5% nonfat dry milk in TBS-T at 37°C for 1 hour.

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L Na2 citrate) for 20 minutes and once with 1× SSC/1% SDS for 20 minutes. The filters were exposed to an Amersham Hyperfilm MP film with an intensifying screen at -80°C.

cDNA probes. The following cDNA fragments were used as probes in the hybridization experiments: a 1.4-kb BamHI-Xba I fragment of a human RARβ cDNA, which was synthesized by polymerase chain reaction (PCR) in the laboratory of Dr P. LeMotte, (Hoffmann-LaRoche, Basel, Switzerland); a 1.9-kb Bgl II fragment of the human t-PA cDNA; and a 1.2-kb Pst I fragment of a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (kindly provided by Dr R. Offringa, State University Leiden, The Netherlands).

Assays. t-PA antigen and PAI-1 antigen determinations were performed by commercially available immunoassay kits. The enzyme immunoassay for determination of human PAI-1 antigen detects active and "latent" (inactive) forms of PAI-1, whereas t-PA/PAI-1 complexes are recovered with about 10-fold lower efficiency.

Reverse transcriptase-PCR (RT-PCR). RT-PCR was performed under standard conditions following the specifications recommended by the supplier. The primers used are specified in Fig 1A. In short, RARβ and actin cDNAs were synthesized in one reaction mixture containing 0.25 μg total RNA, 1.6 μg RARβ cDNA primer (Fig 1A), 0.5 μg oligo dN primer and RT-II-superscript (Life Technologies, Paisley, UK); then, the cDNAs were heated for 8 minutes at 95°C. Subsequently, the cDNAs were treated with RNase H (25 U/mL) for 25 minutes at 37°C. Next, the RARβ and actin cDNAs (1 μL of a 1× diluted cDNA reaction mixture) were amplified in the presence of 5% (vol/vol) DMSO and 5% W-1 (vol/vol; Life Technologies). The amplifications were performed for 30 cycles. The denaturation was performed during 20 seconds at 94°C. Primer extension was performed for 90 seconds at 45°C for the first 5 cycles and at 55°C thereafter. The DNA-synthesizing step was performed at 72°C for 3 minutes. Aliquots of the PCR reaction mixture were separated on an agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator.

Western blotting. For the detection of RARβ protein, nuclear extracts were prepared from HUVECs as described previously by Andrews and Faller with a few modifications. Briefly, cells were washed with phosphate-buffered saline and then allowed to swell on ice for 15 minutes in a buffer containing 10 mmol/L HEPES (pH, 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, and a mixture of proteinase inhibitors (leupeptin, aprotonin, pepstatin, antipain, and chymostatin) at a final concentration of 5 μg/mL. Next, the cells were lysed by pressing them through a needle and then were centrifuged at 13,000 rpm in a Microfuge centrifuge for 30 minutes. After resuspending the nuclei in a buffer containing 20 mmol/L HEPES (pH, 7.9), 25% (vol/vol) glycerol, 0.42 mol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L dithiothreitol, and the mixture of proteinase inhibitors (final concentration, 5 μg/mL), the suspension was rotated for 30 minutes at 4°C. Finally, the membranes were pelleted by centrifugation for 5 minutes at 13,000 rpm in the Microfuge centrifuge, and the supernatant was collected. The protein concentration of the supernatant was determined with the Bradford protein assay (Biorad, München, Germany). Aliquots containing 6 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions using a 7.5% (wt/vol) polyacrylamide gel. Proteins were transferred to nitrocellulose electrophoretically using a semi-dry blotting device. Next the blots were stained with "Ponceau" (Sigma) to check for equal loading and blotting efficiency. Then, the blots were treated with rabbit polyclonal antibodies raised against the F region of human RARα (RP/F; antibodies kindly provided by Prof P. Chambon, Strasbourg, France). As a second antibody, goat antirabbit antibody (Nordic, Tilburg, The Netherlands) conjugated to horseradish peroxi-

dase was used. Detection was performed using the enhanced chemiluminescent detection method (Amershann) according to the manufacturer’s instructions.

RESULTS

Onset of t-PA and RARβ mRNA induction by RA. Figure 2 shows one representative time course (of the three performed) of t-PA and RARβ mRNA induction in HUVECs treated with 1 μmol/L RA for various periods up to 12 hours. t-PA mRNA levels start to increase after about 8 hours, reaching a 3-fold increase over control levels after 12 hours. Before any exposure to RA, HUVECs express very low levels of two transcripts, 3.2 and 3.4 kb, for RARβ. Both transcripts are already increased 6.5- and 7.5-fold, respectively, after 4 hours of incubation with RA and continue to increase to about 11- and 8-fold stimulation, respectively, after 12 hours. As in many other cell types, the expression of RARα, RARγ, RXRa, and RXRβ in HUVECs did not change appreciably on incubation with RA (see also Kooistra et al).

Effect of protein synthesis inhibition on the induction of t-PA and RARβ mRNA by RA. To assess the importance of ongoing protein synthesis on the induction of t-PA mRNA by RA in HUVECs, cells were stimulated with 1 μmol/L RA in the presence of the protein synthesis inhibitor, CHX (5 μg/mL; added 1 hour before the start of the experiment). After 8 hours total RNA was isolated and analyzed by Northern blotting. As shown in Fig 3, CHX (which inhibited protein synthesis up to about 95%; data not shown) completely blocked the induction of t-PA mRNA by RA. In contrast, CHX did not prevent the RA-stimulated increase in RARβ mRNA. The fact that the induction of RARβ by RA is rapid and CHX-resistant is in agreement with findings in other cell types in which it was shown that induction of RARβ by RA is directly mediated by RARα. The induction of t-PA apparently requires the synthesis of an intermediary regulatory protein, which explains the slower onset of the t-PA mRNA increase as compared with that of RARβ mRNA.

Role of RARβ in t-PA induction. Because the induction profile of RARβ preceded that of t-PA (Fig 2), we examined the possibility that the intermediary factor postulated above is RARβ. In a first approach to verify this hypothesis, we designed the following experiment. HUVECs were pretreated for 8 hours with 5 μg/mL CHX and 1 μmol/L RA. This will lead to an increase in RARβ mRNA levels without affecting t-PA mRNA levels (see Fig 3). If RARβ is truly involved in t-PA gene expression, subsequent incubation of these pretreated cells with RA in the absence of CHX should induce t-PA synthesis much faster than it would in control cells, in which RARβ first needs to be induced. Moreover, this undelayed t-PA induction in the pretreated cells should be suppressible with an RAR antagonist. As shown in Fig 4 for one representative experiment of three performed, the addition of 10 nmol/L RA to the RA/CHX-pretreated cells resulted in a 5-fold increase in t-PA synthesis during an 8-hour incubation period, whereas this RA concentration had a negligible effect on t-PA production in HUVECs preincubated with control incubation medium. This undelayed t-PA
induction by RA in the RA/CHX-pretreated HUVECs could be largely suppressed by the simultaneous addition of 10 µmol/L Ro 41-5253, a retinoid that antagonizes the transactivation of RARs by RA. Ro 41-5253 also strongly quenched the t-PA production in RA/CHX-pretreated HUVECs that were subsequently incubated with control medium, suggesting that the relatively high t-PA production by these cells might be caused by residual RA from the pretreatment period. Therefore, we also evaluated t-PA synthesis in HUVECs pretreated with 5 µg/mL CHX alone, a condition also shown to increase RARβ mRNAs, albeit to a lesser extent than that in combination with RA (Fig 3). In this case, incubation of the pretreated cells with control medium hardly

![Fig 2. Time course of t-PA and RARβ mRNA induction by RA. HUVECs were incubated for the indicated time periods with 1 µmol/L RA (+) or RA vehicle, viz 0.01% (vol/vol) DMSO (−). RNA was isolated and analyzed by Northern blot analysis (15 µg/lane) using 32P-labeled probes for t-PA (A), RARβ (B), and GAPDH (C).](image)

![Fig 3. Effect of protein synthesis inhibition on the induction of t-PA and RARβ mRNA by RA. Cells were incubated with or without RA (1 µmol/L) in the absence or presence of CHX (5 µg/mL). CHX was added 1 hour before; control medium contained 0.01% (vol/vol) DMSO. After 8 hours, RNA was isolated and analyzed by Northern blot analysis using 32P-labeled probes for t-PA (A), RARβ (B), and GAPDH (C). This is a representative experiment out of four performed.](image)

![Fig 4. Effect of RA on t-PA antigen production in cells with elevated RARβ mRNA levels and in the presence or absence of RAR antagonist (Ro 41-5253). HUVECs were pretreated for 8 hours with control medium (Con) (0.1% [vol/vol] DMSO), with CHX (5 µg/mL) and RA (1 µmol/L), or with CHX alone (5 µg/mL) to increase RARβ mRNA levels. After washing the cells 3 times with DMEM to remove residual CHX and/or RA, HUVECs were incubated for 8 hours with control medium (0.1% [vol/vol] DMSO; Con), 10 nmol/L RA (RA), 10 µmol/L Ro 41-5253 (Ro), or 10 nmol/L RA and 10 µmol/L Ro 41-5253 (RA + Ro). t-PA antigen levels in the conditioned media were determined with the t-PA enzyme-linked immunosorbent assay as described in Materials and Methods. The data shown are from one representative experiment of three performed and are expressed as mean values of a duplicate experiment, with ranges indicated by error bars.](image)
affected t-PA synthesis; on addition of 10 nmol/L RA, a qualitatively similar response was observed as that described above for the RA/CHX-pretreated cells, ie, a 2.5-fold increase in t-PA production that could be suppressed to a large extent by 10 µmol/L Ro 41-5253. Taken together, these results are consistent with a role of RARβ as a mediator of t-PA induction by RA in HUVECs.

Identification of RARβ isoforms. To find more conclusive evidence for a role of RARβ in the stimulation of t-PA synthesis by RA, we wished to lower specifically RARβ mRNA levels and translation by using antisense oligo dTs. Therefore, it was necessary to identify the RARβ isoforms expressed in HUVECs. As shown in Fig 3, RA treatment of HUVECs induces two mRNA transcripts of 3.4 and 3.2 kb in size, respectively. Western blot analysis showed one specific RARβ protein band with a molecular weight comparable with that of RARβ expressed in transfected COS cells (about 55 kD); the intensity of the RARβ band increased after RA treatment of HUVECs (Fig 5A). Four different isoforms of RARβ are known, which only differ in their N-terminal A domain.24 In contrast to RARβ1 and RARβ3, RARβ2 and RARβ4 (a spliced variant of RARβ2) contain an RA-responsive promoter,22,24,25 suggesting that the two induced RARβ transcripts in HUVECs are the β2 and/or β4 isoforms. Application of RT-PCR analysis, which is able to distinguish between the different isoforms (see Fig 1C), showed predominantly the presence and induction of RARβ2 mRNA in HUVECs and did not show, or showed only to a minor extent, the presence of RARβ4 mRNA (Fig 5B). Therefore, the two inducible RARβ bands observed by Northern blot analysis are likely to predominantly represent two RARβ2 transcripts.

Fig 5. (A) Western blot analysis of RARβ protein in control and RA-treated cells. HUVECs were incubated for 12 hours with vehicle (0.01% [vol/vol] DMSO) or RA (1 µmol/L). Nuclear extracts were prepared from the cells as described in Materials and Methods. As a positive control, nuclear extracts of COS cells transfected with a RARβ expression vector were used. Equal amounts (6 µg) of endothelial nuclear extracts were subjected to electrophoresis on a 7.5% polyacrylamide gel, transferred to nitrocellulose, incubated with a polyclonal antibody against RARβ, and followed by incubation with a second (goat anti-rabbit) antibody conjugated to horseradish peroxidase. Bands could be visualized by adding substrate. The RARβ protein band is visible at 55 kD. The (slower migrating) intense band and some of the minor bands were also observed with higher concentrations of COS cells with or without an RARβ expression plasmid and are considered to be aspecific. One representative experiment of three performed is shown. (B) Induction of RARβ2 and RARβ4 mRNA by RA as determined by RT-PCR. After incubation of HUVECs for 12 hours with vehicle (0.01% [vol/vol] DMSO) or RA (1 µmol/L), RNA was isolated. RARβ cDNA was synthesized using 0.25 µg total RNA and a specific RARβ primer (see Fig 1A) as described in Materials and Methods. The cDNAs were amplified for 30 cycles using the sense primer 5’-AACTTGGGATCTTTCTGGAAC-3’ and antisense primer 5’-CTGGGGAATGTGGTAGTAGCAG-3’ (see also Fig 1A) for 20 seconds at 94°C, for 90 seconds at 45°C in the first 5 cycles and at 55°C in the last 25 cycles, and for 3 minutes at 72°C. The expected length of the PCR fragment of RARβ2 is 1641 nucleotides and that of RARβ4 is 1286 nucleotides. The data presented are representative of four independent experiments.

Inhibition of RA-stimulated expression of t-PA by an antisense oligo dN against RARβ2 mRNA. Because RARβ2 mRNA appeared to be the major form induced by RA in HUVECs, we chose to inhibit RARβ levels by an antisense oligo dN directed specifically against the translation start site of RARβ2 mRNA, following the protocol described in the Materials and Methods section. Besides blocking transla-
Fig 6. Effect of antisense oligo dNs directed against RARβ2 on RARβ2 mRNA levels as determined by RT-PCR. RNA was isolated from the cells, and cDNAs were made as described in Materials and Methods. RARβ2 (A) and actin (B) cDNAs were amplified by PCR using the primers as shown in Fig 1A. Lanes 1 through 6 represent untreated cells, and lanes 7 through 12 are RA-stimulated (1 μmol/L) cells. The following additions were made, with the final concentration in brackets: lanes 1 and 7, none; lanes 2 and 8, random oligo dNs (30 μmol/L); lanes 3 and 9, antisense RARβ4 oligo dNs (30 μmol/L); lanes 4 and 10, antisense RARβ2 oligo dNs (30 μmol/L); lanes 5 and 11, random oligo dNs (60 μmol/L); and lanes 6 and 12, antisense RARβ2 and RARβ4 oligo dNs (both 30 μmol/L).

**Fig 6. Effect of antisense oligo dNs directed against RARβ2 on RARβ2 mRNA levels as determined by RT-PCR.** RNA was isolated from the cells, and cDNAs were made as described in Materials and Methods. RARβ2 (A) and actin (B) cDNAs were amplified by PCR using the primers as shown in Fig 1A. Lanes 1 through 6 represent untreated cells, and lanes 7 through 12 are RA-stimulated (1 μmol/L) cells. The following additions were made, with the final concentration in brackets: lanes 1 and 7, none; lanes 2 and 8, random oligo dNs (30 μmol/L); lanes 3 and 9, antisense RARβ4 oligo dNs (30 μmol/L); lanes 4 and 10, antisense RARβ2 oligo dNs (30 μmol/L); lanes 5 and 11, random oligo dNs (60 μmol/L); and lanes 6 and 12, antisense RARβ2 and RARβ4 oligo dNs (both 30 μmol/L).

1 2 3 4 5 6 7 8 9 10 11 12
- - - - - + + + + + +

RA (1 μmol/L)

A

B

**Fig 6. Effect of antisense oligo dNs directed against RARβ2 on RARβ2 mRNA levels as determined by RT-PCR.** RNA was isolated from the cells, and cDNAs were made as described in Materials and Methods. RARβ2 (A) and actin (B) cDNAs were amplified by PCR using the primers as shown in Fig 1A. Lanes 1 through 6 represent untreated cells, and lanes 7 through 12 are RA-stimulated (1 μmol/L) cells. The following additions were made, with the final concentration in brackets: lanes 1 and 7, none; lanes 2 and 8, random oligo dNs (30 μmol/L); lanes 3 and 9, antisense RARβ4 oligo dNs (30 μmol/L); lanes 4 and 10, antisense RARβ2 oligo dNs (30 μmol/L); lanes 5 and 11, random oligo dNs (60 μmol/L); and lanes 6 and 12, antisense RARβ2 and RARβ4 oligo dNs (both 30 μmol/L).

LIGAND DEPENDENCY OF THE RARα- AND RARβ-MEDIATED STEPS IN t-PA INDUCTION

We next addressed the question of whether each of the two steps putatively involved in t-PA induction by RA, namely induction of RARβ via RARα followed by t-PA induction via RARβ, requires different ligand concentrations for activation. To that end, we assessed the relative potencies of RA, 9-cis RA, and combinations thereof on the induction of RARβ2 mRNA in control HUVECs and on the induction of t-PA mRNA in cells with elevated RARβ levels by pretreatment with CHX. As shown in Fig 8A, for an 8-hour incubation period, RA and 9-cis RA were equipotent in inducing RARβ mRNA; both compounds required a minimal concentration of 100 nmol/L to induce RARβ mRNA, and induction levels increased to about 9-fold stimulation at 1 μmol/L with both ligands. A combination of the two isomers showed at least an additive effect on the expression of RARβ mRNA at suboptimal concentrations but never exceeded the maximal stimulation induced by either RA or 9-cis RA alone. At supramaximal concentrations (10 μmol/L RA or a combination of 1 μmol/L RA and 1 μmol/L 9-cis RA), RARβ mRNA induction levels decreased again. In contrast to the induction of RARβ, RA had already induced t-PA mRNA strongly at concentrations as low as 10 nmol/L in CHX-pretreated cells, and this response was only slightly more increased at concentrations of 1 μmol/L (Fig 8C). 9-cis RA was much less effective than RA. At least 10-fold higher concentrations of 9-cis RA were required to achieve comparable induction of t-PA mRNA. In general, RA in combination with 9-cis RA was not more effective than RA alone in enhancing t-PA mRNA induction. These results indicate that each of the two steps involved in t-PA mRNA induction responds differently to (combinations of) different concentrations of RA and 9-cis RA, and that RA and 9-cis RA are not equipotent in inducing t-PA expression.

DISCUSSION

In a previous study, we have reported on the involvement of RARα in the induction of t-PA gene expression by RA in HUVECs. However, the relatively slow action of RA on t-PA gene expression suggested a mechanism in which the induction of t-PA is a secondary response to activation of RARα by RA. We now provide evidence that a second step is indeed necessary and involves the synthesis of RARβ2 on the basis of the following findings: (1) the kinetics of the RA-associated increase in RARβ mRNA are consistent with the subsequent involvement of RARβ in the regulation of t-PA; (2) the effect of RA on the induction of t-PA, but not that of RARβ, was completely abolished by the protein synthesis inhibitor, CHX; (3) HUVECs with elevated RARβ mRNA levels as a result of pretreatment of the cells with RA and/
or CHX showed an undelayed increase in t-PA expression by RA that could be quenched by the RAR antagonist Ro 41-5253; and (4) antisense oligo dNs directed against RARβ2 greatly suppressed the induction of t-PA by RA. Taken together, these results point to a 2-step mechanism in which RARβ2 is first induced via RARα and then RARβ2 mediates the induction of t-PA. Experiments with different concentrations of RA and 9-cis RA showed that each step has a different ligand dependency.

Complementary to our studies, Bulens et al.13 identified a functional RARE in the t-PA promoter, consisting of a direct repeat of the GGCTCA motif spaced by 5 nucleotides (DR5) and localized at −7.3 kb. Transient expression of a 2-kb RARE-containing t-PA promoter:CAT construct into the hybrid endothelial cell line EA.hy926 resulted in a 6-fold induction by RA, whereas no induction was observed with a reporter construct in which the t-PA DR5 RARE was eliminated by site-specific mutagenesis. A maximal induction by RA of this RARE-containing t-PA promoter:CAT construct in the endothelial cell line required cotransfection with RARβ- and RXRa-expressing plasmids. Our results in combination with those of the study by Bulens et al.13 provide strong evidence that RARβ2 is the ultimate mediator of t-PA induction by RA in HUVECs.

A 2-step mechanism as described above for the induction of t-PA by RA in HUVECs is not unique in itself for retinoid-induced genes. For example, similar observations were made with some retinoid-responsive genes in F9 cells, such as laminin B1, collagen IV, and J6 genes.26 These genes were induced after a relatively long (12 to 24 hours) exposure to retinoids, and their induction was also sensitive to CHX. Also, the relatively slow action of RA on transferring and albumin gene expression in Hep3B cells (8 to 24 hours) may be a secondary response to RA.27 Several factors have been shown to serve as an intermediary protein in the RA response, including c-Jun, GATA-binding proteins, and nuclear factor κB heterodimers.28-30 However, the number of RA-responsive genes whose induction is known to be mediated by an RAR as a secondary protein, such as the t-PA induction in HUVECs in this study, is still rather limited. To our knowledge, only the induction of major histocompatibility complex class I in NTera-2 embryonal carcinoma cells has been shown to be at least partly dependent on the RA-induced increase of RARβ.30

Our results do not exclude the possibility that, in addition to RARβ2, other factors are required for the induction of t-PA by RA. Several reports have provided evidence that members of the steroid/thyroid receptor superfamily, including retinoid receptors, require cofactors for transcription activation. For example, Folkers and Van der Saag21 have shown that the adenovirus E1A protein functions as a cofactor for RARβ-mediated activation of transcription. Also, functional interactions of peroxisome proliferator-activated receptor, RXR, and SPI in the transcriptional regulation of the acylcoenzyme-A oxidase promoter have been shown.32 Whether RARβ2 also requires a cofactor for induction of t-PA by RA is uncertain. However, it may be significant, as discussed by Bulens et al.13 that, in the vicinity of the RARE in the t-PA promoter, several SPI and AP2 consensus binding sites are present.

Our results support initial suggestions that the various RAR subtypes may preferentially control the transcription of different subsets of RA-responsive genes. This concept was based on two types of observations: (1) the striking interspecies amino acid sequence conservation of a given RAR (and RXR) isoform, which is much higher than the similarity between the three RAR (or the three RXR) isoforms within a given species31; and (2) the specific spatiotemporal patterns of expression for each RAR (and RXR) gene as shown by in situ hybridization studies on embryo sections.33-35 More direct evidence came from cotransfection experiments in which each of the RAR and RXR isoforms showed some specificity towards several synthetic and natu-

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**Fig 7.** Effect of antisense oligo dTs directed against RARβ2 mRNA on the induction of t-PA and PAL1 synthesis by RA. HUVECs were incubated with or without RA (1 μmol/L) in the presence of antisense oligo dTs directed against RARβ2 (β2) and/or RARβ4 (β4) at the indicated concentrations. As controls, medium with (Ran) or without (Con) random antisense oligo dTs was used. Oligo dTs were added 1 hour before and 4 hours after the addition of RA at the indicated concentrations. After 12 hours, t-PA (A) and PAL1 (B) levels in the conditioned media were measured with the enzyme-linked immuno-sorbent assay procedures as described in Materials and Methods.
ral RAREs. Also, in a different type of study, targeted disruption of RARα in F9 cells resulted in reduced cellular RA-binding protein II (CRAPBPII) and Hoxb1 mRNA expression in RA-stimulated cells. However, it has been found that a certain degree of redundancy may exist in the retinoid-signaling pathway. For example, knock-out mice for all RARα isoforms showed a phenotype that is relatively discrete considering the ubiquitous expression of RARα.

and knock-out mice for specific isoforms studied so far (RARα1, RARβ2, and RARγ2) have no apparent phenotype. Similarly, some of the natural promoters that have been tested in cotransfection experiments could be activated, albeit to different extents, by more than one RAR or RXR form. Also in the case of t-PA, Bulens et al. showed that RARα, RARβ, and RXRα were all able to stimulate a 2.4-kb RARE-containing t-PA promoter fragment linked to a

Fig 8. (A) Induction of RARβ mRNA by RA, 9-cis-RA, or a combination thereof. HUVECs were incubated for 8 hours with the indicated concentrations of retinoids. RNA was isolated and analyzed (15 μg/lane) by Northern blot analysis using [deoxycytidine triphosphate]-labeled probes for RARβ and GAPDH. (B) Quantification of RARβ mRNA. The signal intensity of the Northern blot was determined by scanning and was normalized to the signal from the internal standard GAPDH. The amount of RARβ mRNA is expressed relative to the amount of control medium. (C) Stimulation of t-PA mRNA synthesis by RA, 9-cis RA, or a combination thereof in cells with elevated RARβ levels. HUVECs were preincubated for 8 hours with CHX (5 μg/ml) to increase RARβ mRNA levels. After washing the cells 3 times with DMEM, the cells were stimulated for another 8 hours with the indicated concentrations of RA, 9-cis RA, or both compounds. Total RNA was isolated and hybridized (15 μg/lane) to the [deoxycytidine triphosphate]-labeled t-PA and GAPDH probes. (D) Quantification of t-PA mRNA. The signal intensity of the Northern blot was determined by scanning and normalized to the signal from the internal standard GAPDH. The amount of t-PA mRNA is expressed relative to the amount of control medium.
CAT reporter gene in cotransfection experiments in HT1080 fibrosarcoma cells, although RARβ in combination with RXRα gave maximal stimulation in these experiments. However, in these studies transfected cells have been used expressing concentrations of RARs/RXRα at nonphysiologically high levels. Therefore, these transfection experiments do not necessarily imply that the same regulatory mechanisms hold true under physiological conditions; ie, restricted receptor expression is one mechanism of specifying gene activation by hormones. Our results obtained in HUVECs indicate that, in a physiological context, RARα specifically induces RARβ and that the RAR subtype β2 is involved in the induction of t-PA, which points to a preference of different RAR subtypes to induce different target genes.

It is assumed that, in the cellular context, RARs act predominantly as heterodimers with RXRs.43 Our results shown in Fig 8 show that RA and 9-cis RA are equipotent in stimulating RARβ expression in HUVECs and that a combination of both ligands never is more effective than each compound alone at optimal concentration. For the induction of t-PA in HUVECs with elevated RARβ levels, RA is even much more effective than 9-cis RA. Because RA acts mainly via binding to RARs, whereas 9-cis RA can bind to and activate both RARs and RXRs,44,45 ligand binding to RXR, if occurring, does not substantially contribute to the two transactivation steps. In line with this conclusion, we found that 4-{1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethyl}benzoic acid, a compound shown to be RXR-specific,45 was only a very weak inducer of RARβ and t-PA mRNA in HUVECs (Lansink and Kooistra, unpublished data). A similar conclusion was reached by Xiao et al,46 who showed that binding of an RXR specific ligand, SR11237, to RXR in RAR-RXR heterodimers did not confer ligand-dependent transactivation of a β-RARE-tk-CAT promoter construct.

A remarkable finding was the observation that higher concentrations of RA are required for the stimulation of RARβ expression, which involves RARα activation, than for the induction of t-PA expression, which involves RARβ activation. Binding studies with purified RARα and RARβ preparations show equal affinity of both receptors for RA.46,49 In agreement with our findings, Brand et al46,49 also reported a 10 times higher apparent affinity of RA for RARβ than for RARα in a cellular system. A similar discrepancy between data from binding studies with purified receptors and data from cell studies exists for RA and 9-cis RA in relation to RARβ. Our results show that 9-cis RA is less potent in inducing t-PA than is RA, although binding studies using isolated receptors show similar binding affinities of RA and 9-cis RA for RARβ.46,49 Apparently, cellular factors influence the binding affinities of retinoids for their receptors.

Our observation that RARβ transcripts in HUVECs are upregulated by retinoid treatment is not just a cell culture phenomenon, because both a 3.3-kb and a 3.0-kb RARβ mRNA are increased within 1 to 4 hours in lung and liver tissues from retinol-deficient rats receiving a single dose of RA (100 μg).51 However, under normal physiological conditions, RA concentrations in plasma and tissues are sufficient to maintain a steady RARβ expression.52 Also, because of the high affinity of RARβ for RA (Brand et al46 and this study), it seems that the capacity of RA to stimulate t-PA synthesis in vivo is almost fully exploited under normal physiological conditions. In line with this finding, plasma t-PA activity in vitamin A-deficient rats is about 3-fold lower than control values, whereas RA treatment of normal rats increases plasma t-PA activity maximally by only 50%.50 Similarly, treatment of male volunteers with isotretinoin (13-cis RA), which has been shown to induce t-PA levels in HUVECs,51 did not effect or only minimally effected t-PA antigen in plasma.52,53 Therefore, attempts to increase plasma t-PA levels by the use of retinoids should be directed at finding RARβ-specific ligands with a higher transactivation capacity than that of RA, rather than at increasing plasma RA levels.

Taken together, our results provide evidence that the induction of t-PA synthesis by RA in HUVECs occurs via a 2-step process in which one receptor subtype, RARα, is required for the induction of a second subtype, RARβ2, that subsequently mediates t-PA synthesis. These apparently subtype-specific functions of RARs in HUVECs are accompanied by a different ligand dependency of each step; whereas RA and 9-cis are equipotent in inducing RARβ, RA shows a much higher efficacy than 9-cis RA in inducing t-PA synthesis in cells with elevated RARβ levels.

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