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Transcriptional activation of endothelial cell integrin αv by protein kinase C activator 12(S)-HETE

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SUMMARY

Previous work demonstrated that 12(S)-HETE [12(S)-hydroxyeicosatetraenic acid], a lipoxygenase metabolite of arachidonic acid, stimulates the surface expression of integrin αvβ3 on mouse lung vascular endothelial cells (CD clone 3) in a post-transcriptional and protein kinase C (PKC)-dependent fashion. In this study we examined the effect of 12(S)-HETE on the expression of integrin receptors αvβ3 and α5β1 in a different clone of a mouse endothelial cell population derived from lung microvasculature (designated CD clone 4). The results indicated that 12(S)-HETE transcriptionally activates the gene expression of integrin αv as assessed by quantitative reverse transcription/polymerase chain reaction/Southern hybridization, RNase protection assay, solution hybridization, and northern blotting. The induction of αv mRNA occurred within 1 hour, peaked at ~4 hours (2- to 4-fold increase), persisted for up to 16 hours, and thereafter gradually declined. The PKC activator phorbol 12-myristate 13-acetate (PMA) induced the αv mRNA, in a similar way. 12(S)-HETE treatment did not, in contrast, alter the mRNA levels of integrin subunit α5 or β1. The induction of αv mRNA appeared to be protein synthesis-independent, since cycloheximide did not alter the 12(S)-HETE effect. 12(S)-HETE also did not appear to alter the mRNA half-life of αv. On the other hand, 12(S)-HETE-induced increase in αv mRNA levels was PKC-dependent, since pre-treatment of CD clone 4 cells with calphostin C significantly inhibited 12(S)-HETE-increased αv mRNA. Nuclear run-off experiments revealed that the increase in αv mRNA results from an enhanced gene transcription. Facilitated αv gene transcription resulted in an increased surface expression of αvβ3 protein, which resulted in an increased cell adhesion to vitronectin. The above observations, in conjunction with our previous experimental data, suggest that 12(S)-HETE may employ diverse mechanisms to stimulate the integrin αvβ3 expression in vascular endothelial cells, which could play important roles in tumor cell adhesion, angiogenesis, hemostasis, and many other vascular events.

Key words: PKC, integrin αv, 12(S)-HETE, mRNA

INTRODUCTION

Vascular endothelial cells express several integrin receptors including α1-6/β1, αvβ1, αvβ3, αvβ5, and α6β4 (Albelda et al., 1989; Cheng and Kramer, 1989; Cheresh, 1987; Klein et al., 1993; Massia and Hubbell, 1992). These α/β heterodimers, together with some other adhesion molecules such as cadherins and PECAM-1 (platelet endothelial cell adhesion molecule-1), play an essential role in the formation and maintenence of endothelial cell monolayers and in linking endothelial cells to extracellular matrix (Lampugnani et al., 1991; Tang et al., 1993c). Quantitatively, αvβ3 and α5β1 are the two major integrins expressed by endothelial cells. Although α5β1 has been shown to primarily mediate endothelial cell adhesion to fibronectin, αvβ3 is capable of mediating endothelial cell adhesion to a wide diversity of ligands including fibronectin, vitronectin, von Willebrand factor, fibrinogen, osteopontin, thrombospondin, denatured collagen, and perlecan (Cheng et al., 1991; Dejana et al., 1988; Felding-Habermann and Cheresh, 1993). The difference in the spectra of binding substrata, between αvβ3 and α5β1, suggests that the former may be functionally more versatile and important in various adhesive interactions involving endothelial cells and other cell types as well as endothelial cells and matrix proteins. Our series of studies on microvascular endothelial cells stimulated by 12(S)-HETE indicate that this may be the case.

12(S)-HETE [12(S)-hydroxyeicosatetraenoic acid] is a hydroxy fatty acid derived from the lipoxygenase metabolism of arachidonic acid. Exogenous 12(S)-HETE induces a reversible retraction of endothelial cells (i.e. CD clone 3 lung-derived endothelial cells) which involves a well-coordinated reorganization of cytoskeletal proteins and integrin αvβ3 but not α5β1 (Tang et al., 1993c), suggesting that αvβ3 integrin is more important in maintaining the integrity of endothelial cell monolayers. In fact, only αvβ3, not α5β1, is found to be localized to the cell-cell borders of CD clone 3 cultures (Tang et al., 1993c). 12(S)-HETE promotes tumor cell adhesion to both large-vessel (Tang et al., 1993b) and microvascular (Tang

et al., 1994) endothelial cells, which is mediated by upregulated expression of αvβ3 but not α5β1 on endothelial cells. The 12(S)-HETE-enhanced surface expression of αvβ3 in CD clone 3 cells is found to be a posttranscriptional process dependent on protein kinase C (PKC) activation and structural integrity of cytoskeletal elements (Tang et al., 1994).

In light of the distinct effects of 12(S)-HETE on endothelial cell αvβ3 integrin, we screened by quantitive reverse transcription PCR (RT-PCR) several endothelial cell lines derived from rat or mouse aorta, lung, liver, spleen, and kidney for the expression of α5β1 and αvβ3, following stimulation by 12(S)-HETE. Interestingly, in a different cloned endothelial cell line (CD clone 4) also established from murine lung microvasculature, 12(S)-HETE was observed to increase the mRNA levels of αv. This paper presents data characterizing the 12(S)-HETE-stimulated gene transcription of αv in CD clone 4 cells.

MATERIALS AND METHODS

Endothelial cell culture and characterization

Mouse lung-derived endothelial cells (CD clone 3) were established, cloned and characterized as described previously (Chopra et al., 1990). These endothelial cells have been used extensively in our previous experiments and many cellular properties such as integrin expression have been well characterized (Tang et al., 1993c, 1994). The CD clone 4 endothelial cell line was established and cloned in a similar manner. These cells were initially characterized at the morphological level in the same way as for CD clone 3 cells. Additional characterization was achieved by immunofluoresecnt staining with antibodies directed against various cytoskeletal proteins, integrins, and endothelial cell markers (Table 1; Fig. 1) to confirm the endothelial nature of this cell population. Both CD clone 3 and clone 4 cells were cultured and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Gibco; Grand Island, NY) and 0.05% gelatin in a 5% CO_2 humidified atmosphere. Cells were subcultured routinely three days post-confluency to maintain the phenotypic stability of the line. Cells were passaged by a mixture of trypsin (0.1%) and 2 mM EDTA. The endothelial cell phenotype was regularly examined as described previously (Diglio et al., 1989, 1993).

Eicosanoids, chemicals and antibodies

12(S)-HETE was purchased from Cayman Chemical (Ann Arbor, MI) and used to treat cells at a final concentration of 0.1 μM unless otherwise specified. Rabbit anti-human αvβ3 and α5β1, and mAb against αv and αvβ5 (P1F6), were obtained from Gibco Life Technologies. A mAb to β3, OPG-2, was generously provided by Dr T. J. Kunicki (The Blood Center, WI). Some other antibodies used to characterize the CD clone 4 endothelial cells were listed in Table 1. Fluorescein (FITC)-conjugated goat anti-rabbit IgG was purchased from ICN (Lisle, IL). PKC inhibitor, calphostin C (the IC_{50} values are: for PKC, 0.05 μM; for PKA>50 μM; for cGMP-dependent protein kinase>25 μM; and for myosin light chain kinase>5 μM), was isolated from *Cladosporium cladosporioides* (Kamiya Biochem Co., Thousand Oaks, CA). The protein kinase A (PKA) inhibitor, H8 (the IC_{50} values are: for PKC, 15 μM; for PKA, 1.2 μM; for cGMP-dependent protein kinase, 0.48 μM; and for myosin light chain kinase, 68 μM), was purchased from Seikagaku America, Inc. (St. Petersburg, FL). Genistein, cycloheximide, and actinomycin D were all purchased from Sigma. The MaxiScript in vitro transcription kit and RPA II ribonuclease protection kit were purchased from Ambion (Austin, TX). Nonimmune rabbit IgG (Cooper Biochemical, Malvern, PA) and MOPC 21 tumor cells ascites (IgG1; Sigma) were used as polyclonal and monoclonal antibody controls, respectively. Goat whole serum (Sigma) was used to block nonspecific Fc receptors.

Table 1. Antibodies used in this study

Antigen	Nature of the Ab	Source
Vimentin	Goat pAb (77-110-2)	ICN
Cytokeratin	Mouse mAb (RPN1165)	Amersham
Desmin	Mouse mAb (RPN1101)	Amersham
α-Actin (smooth muscle specific)	Mouse mAb (MU128-UC)	BioGenex Lab.
αvβ3	Rabbit pAb (12119-012)	Gibco
α5β1	Rabbit pAb (12118-014)	Gibco
αvβ5	Mouse mAb (P1F6)	Gibco
αv	Mouse mAb (VNR147)	Gibco
β3	Mouse mAb (OPG-2)	Dr T. Kunicki
α5	Mouse mAb (P1D6)	Oncogene
β1	Mouse mAb (P4C10)	Gibco
P-selectin	Mouse mAb (JZ-27.380.26)	Accurate
PECAM-1	Rabbit pAb (SEW-3)	Dr P. Newman
vWF	Rabbit pAb (AXL-205/2)	Accurate
vWF	Goat pAb (68-112-1)	ICN

Quantitative RT-PCR, Southern blotting of PCR fragments, and northern blotting

The mRNA levels of several integrin subunits, i.e. α5, β1, αv, β3, were screened with RT-PCR combined with Southern hybridization of the PCR fragments. CD clone 4 endothelial cells (passage 4-10) were treated with either solvent (ethanol) or 0.1 μM 12(S)-HETE in serum-free DMEM for 5 minutes, followed by washing away of the agents. Then cells were returned to serum-containing DMEM to be cultured for various time periods, i.e. 0.5, 1, 1.5, 2, 3, 4, 8, 16, and 24 hours. This treatment protocol has been consistently used in our previous experiments (Honn et al., 1989; Tang et al., 1993b,c, 1994). At the end of treatment, cells were harvested with trypsin/EDTA and total cellular RNA prepared from cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski, 1993) using the Tri-Reagent kit (MRC, Inc., Cincinnati, OH). Poly(A)$^+$ RNA was isolated using a PolyATract RNA kit (Promega; Tang et al., 1993a). RT-PCR was performed basically as previously described using human sequence-based PCR primers (Tang et al., 1993c, 1994). RT and PCR reactions without template (i.e. RNA and cDNA, respectively) were used as the experimental negative control. DNase treatment was used prior to the initiation of RT reaction to prevent false priming off from genomic DNA. Two types of quantitative RT-PCR were performed in the present study. The first, parallel RT-PCR, used external controls such as β-actin and GAPDH for which the RT-PCR was performed simultaneously in separate tubes with the RT-PCR reactions for integrins. RT was performed in 20 μl of mixture containing 1 μg heat-denatured (65°C, 10 minutes) total RNA, 1× RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 75 μg/ml BSA, 25 ng/μl antisense primer, 0.5 mM dNTPs, 30 units RNasin, and 200 units MMLV reverse transcriptase at 42°C for 1 hour. PCR was performed in the GeneAmp 9600 PCR system (Perkin Elmer) and preliminary experiments were conducted to ensure that the PCR amplification was in the exponential range and the amplification efficiency was very similar for different primers. To this end, PCR was terminated at every five cycles, starting from cycle 25, to cycle 45. An aliquot of PCR products was then gel-separated, transferred, hybridized, and the intensity determined by densitometry scanning (see below). The cycle number at which PCR was still in a phase of apparent linear amplification was thus chosen for each pair of primers. PCR was performed in a total volume of 50 μl containing 1 μl of RT-derived cDNA, 1× PCR buffer (30 mM Tris-HCl, pH 8.5, 2 mM $MgCl_2$, 100 μg/ml BSA), 250 nM sense and antisense primers, 200 μM dNTPs, and 1 unit AmpliTaq (Perkin-Elmer) using different cycling profiles for different pairs of primers (Table 2). In the second type of quantitaive RT-PCR, i.e. one-tube RT-PCR, appropriate amounts of αv and β1 primers, or α5 and β3 primers, were mixed with PCR mixture containing also RTase and both RT and PCR

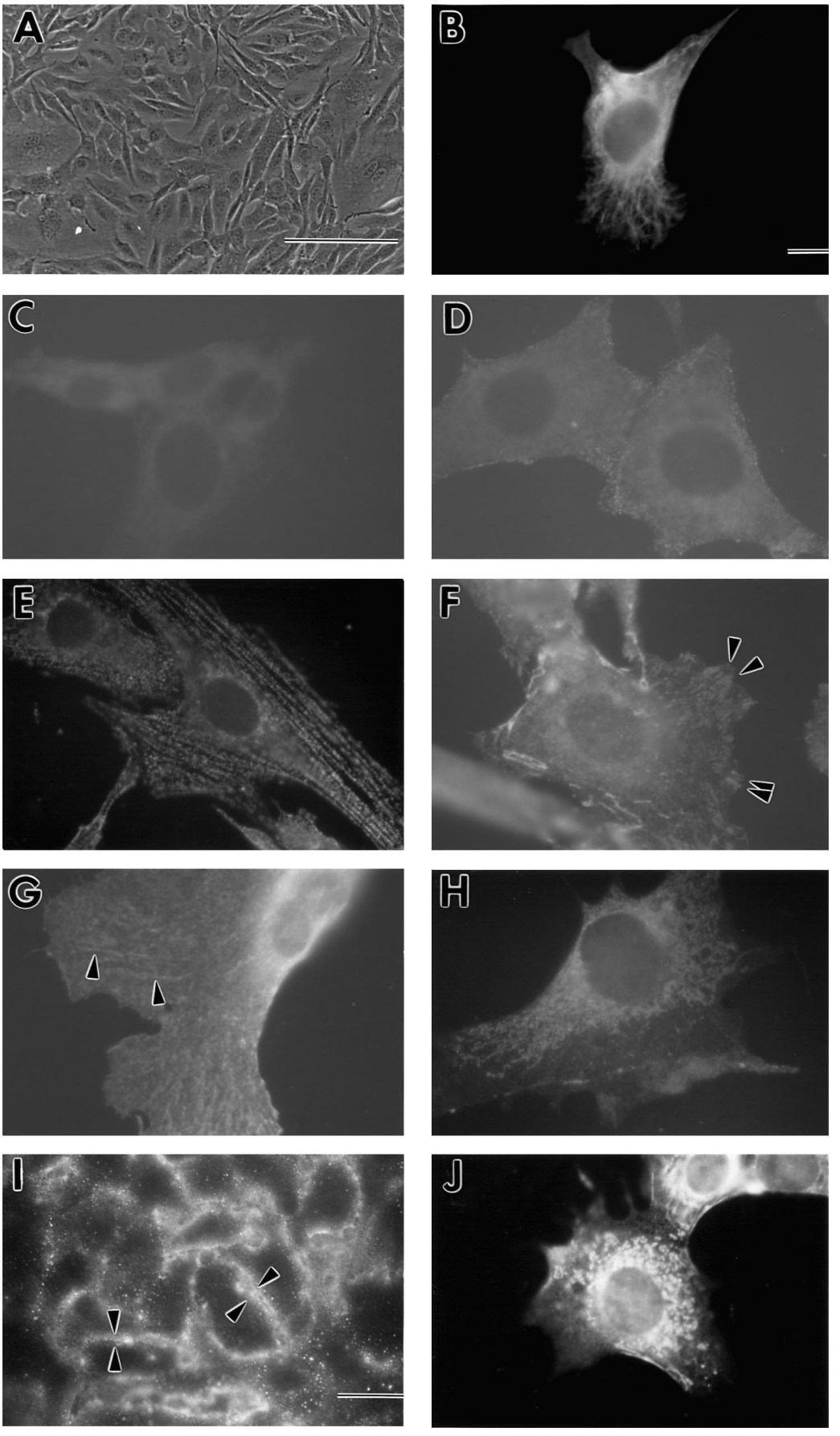


Fig. 1. Immunological characterization of CD clone 4 cells as endothelial cells. (A) Microphotograph showing the morphology of confluent CD clone 4 cell cultures. (B-D) and (F-J) Immunostaining of CD clone 4 cells with antibodies to vimentin (B), cytokeratin (C), desmin (D), αvβ3 (F), α5β1 (G), P-selectin (H), PECAM-1 (I), and von Willebrand factor (J). (E) Murine 3T3 fibroblasts stained with anti-desmin antibody. Immunofluorescent staining with some other antibodies (see Table 1) including smooth muscle cell-specific α-actin is not shown. Arrowheads in F and G indicate faint localization of integrin αvβ3 and α5β1 to focal adhesions. Cells in I used for PECAM-1 staining were cultured on Vitrogen as previously described (Muller et al., 1989) and the arrowheads indicate cell-cell borders. Bars, 10 μm (the magnifications for B-H and J are the same).

Table 2. PCR primers and cycling conditions

Primers	Sequence	Size (bp)	Cycling profile
α5	5′-CATTTCCGAGTCTGGGCCAA-3′ (sense) 5′-TGGAGGCTTGAGCTGAGCTT-3′ (antisense)	323 (nucleotide 2832-3155)	94°C; 45 s 58°C; 45 s 72°C; 1 min 30 cycles total
β1	5′-TGTTCAGTGCAGAGCCTTCA-3′ (sense) 5′-CCTCATACTTCGGATTGACC-3′ (antisense)	452 (nucleotide 2041-2473)	94°C; 1 min 53°C; 1 min 72°C; 1 min 30 cycles total
αv	5′-GTTGGGAGATTAGACAGAGGA-3′ (sense) 5′-CAAAACAGCCAGTAGCAACAA-3′ (antisense)	288 (nucleotide 2787-3054)	94°C; 45 s 52°C, 45 s 72°C; 1 min 25 cycles total
β3	5′-GGGGACTGCCTGTGTGACTC-3′ (sense) 5′-CTTTTCGGTCGTGGATGGTG-3′ (antisense)	521 (nucleotide 1771-2254)	94°C; 1 min 58°C; 1 min 72°C; 1 min 30 cycles total
β-Actin	5′-GTGGGCCGCT(C)CTAGGCACCA-3′ (sense) 5′-GATGGAGCCG(A)CCG(A)ATCCACA-3′ (antisence)	930	94°C; 1 min 50°C; 1 min 72°C; 2 min 30 cycles total
GAPDH	5′-CCACCCATGGCAAATTCCATGGCA-3′ (sense) 5′-TCTAGACGGCAGGTCAGGTCCAC-3′ (antisense)	598	94°C; 1 min 47°C; 1 min 72°C; 1.5 min 30 cycles total

reactions were performed in the same tube according to the following protocol: 42°C for 8 minutes (RT), 94°C for 5 minutes (heating to deactivate RTase), regular PCR (94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute) for 25 cycles, and finally 72°C for 5 minutes (final extension).

To quantify the mRNA levels of these integrin subunits before and after 12(S)-HETE treatment, 20 μl of PCR-amplified cDNA was loaded onto a 1% agarose gel and transferred to GenescreenPlus nylon membrane (NEN, DuPont) using a PosiBlot Pressure Blotter (Stratagene, La Jolla, CA). For northern blotting, 10 μg of mRNA was separated on a 1% formaldehyde/agarose gel and similarly transferred to the nylon membrane. DNA or RNA was UV-linked to the membrane using a Stratalinker UV crosslinker (Stratagene). Specific cDNA probes for human integrin αv, β3, α5, β1, GAPDH, or β-actin (Chang et al., 1991; Tang et al., 1994) were labeled either with fluorescein using the ECL random prime labeling system (Amersham) or with [α-^{32}P]dCTP using the Prime-it random primer labeling kit (Strategene). The fluorescein-labeled probes in conjunction with anti-fluorescein antibody and the ECL detection system were used for Southern hybridization of PCR fragments. The ^{32}P-labeled probes were used for northern blotting. Prehybridization, hybridization, post-hybridization wash, and autoradiography were performed as previously described (Tang et al., 1993a). The intensity of the bands on either Polaroid negative films or autoradiographic films was determined by scanning with an LKB Ultrascan XL laser densitometer.

Ribonuclease protection assays

To confirm the results obtained by quantitative RT/PCR and northern blotting, we performed ribonuclease protection assays. Human integrin (i.e. α5, β1, αv, and β3) cDNA sequences were cloned into pBlueScript SK vectors (Chang et al., 1991) and linearized with various restriction enzymes and the sense and antisense riboprobes prepared using T3 and T7 RNA polymerases, respectively. Specifically, individual α5, β1, αv, and β3 cDNAs were linearized with *Hin*dIII, *Bst*XI, *Sac*I, and *Not*I to generate antisense riboprobes (with T7 RNA polymerase) of 263 bp, 123 bp, 251 bp, and 874 bp, respectively. Similarly, purified α5, β1, αv, and β3 cDNAs were linearized with *Eco*RV, *Hin*dIII, *Xho*I, or *Sac*I to generate (with T3 RNA polymerase) sense riboprobes of 573 bp, 243 bp, 243 bp or 132 bp, respectively. As a control, a murine β-actin DNA template linearized with *Hin*dIII (Ambion, Inc.) was used to generate an antisense probe (with T7 polymerase) with a protected length of 250 bp. The linearized DNA templates were purified with proteinase K/phenol-chloroform extraction and the respective riboprobes synthesized with T7 or T3 RNA polymerases by including [α-^{32}P]UTP in the transcription mixture. The in vitro transcription was performed using the Maxi-Script transcription kit (Ambion, Texas) with the final concentrations being: 500 μM for ATP, GTP, and CTP; 3.125 μM for [α-^{32}P]UTP (800 Ci/mmol); and 5 μM for the cold UTP. The transcription reaction (in a total of 20 μl) was initiated by adding 1 μl (10 units) of RNA polymerase and performed by incubating the mixture at 25°C for 1 hour. At the end of the reaction, the DNA templates were degraded with RNase-free DNase and the run-off transcripts were purified with the Chroma-spin size-exclusion chromatography (Clontech). The quality of the transcribed riboprobes was evaluated by running 1 μg of the purified products on a 3% formaldehyde/MOPS/LMP-agarose gel followed by autoradiography. In all cases, single-band species of the expected sizes were identified for each individual probe. Hybridization and nuclease digestion were performed using a RPA II ribonuclease protection kit (Ambion) according to the manufacturer's instructions except for some minor modifications. A sample of 8-10 μg of total RNA from each condition as described in Results was dried down in a Speedvac and mixed with 1×10^4 to 1×10^6 cpm of each riboprobe and the hybridization buffer. The hybridization was performed at 42°C in a rotating hybridization oven (Bellco Biotechnology) for 24 hours. The hybridization mixture was then digested with RNase and the hybrids precipitated and used for gel running. In some experiments, in order to explore the role of protein synthesis in 12(S)-HETE-modulated integrin transcription, CD clone 4 endothelial cells were pretreated with cycloheximide (50 μM final concentration) for 1 hour followed by 12(S)-HETE (0.1 μM; 4 hours) stimulation in the presence of cycloheximide. In some other experiments, CD clone 4 cells were pretreated with various protein kinase inhibitors including calphostin C (0.5 μM), H8 (5 μM), and genistein (50 μg/ml) for 15 minutes before 12(S)-HETE (0.1 μM) stimulation. In still another set of experiments, CD clone 4 cells were first stimulated with 12(S)-HETE (0.1 μM) or solvent control for 4 hours and then treated with 5 μg/ml of actinomycin D for various time periods of up to 6 hours.

Alternatively, endothelial cells were first treated with actinomycin D (8 hours) followed by 12(S)-HETE (0.1 μM; 4 hours) stimulation in the presence of actinomycin D. In all cases total cellular RNA was extracted using the acidic guanidinium thiocynanate-phenol-chloroform extraction method (Chomczynski, 1993) and equal amounts of RNA from each condition were used in the protection assays. Protected fragments were separated on a 5% denaturing polyacrylamide gels and identified by autoradiography. The intensity of the protected autoradiographic bands was determined by densitometric scanning.

Solution hybridization

CD clone 4 cells were treated with 12(S)-HETE (0.1 μM) or PMA (0.1 μM) for 0, 1, 2, 4, 8, and 24 hours and total RNA was isolated as described above. Sense αv riboprobe and antisense αv or β-actin probes were prepared as detailed above using [^{35}S]UTP (DuPont) as the lablel. One million cpm of αv riboprobes (sense and antisense) or 250,000 cpm of β-actin antisense riboprobe was used in hybridization with 10 μg of total RNA (42°C for 24 hours). For each sample, solution hybridizations to both αv and β-actin were simultaneously performed. After hybridization, the RNase digestion and precipitation of protected fragments were performed as described above for the RNase protection assay. Following washing with cold 75% ethanol and vacuum drying, the pellet was dissolved in 30 μl of sterile water at 65°C. Then 10 μl portions of each sample were collected onto a glass fiber disc, which was washed (4×) with 10% TCA containing 1 mM sodium pyrophosphate. Dried discs were counted for radioactivity in scintillation fluid with a β-counter (Packard). Each condition was run in triplicate and the mean cpm values of αv were normalized against corresponding β-actin cpm values. The results were expressed either as cpm or % of the zero time point.

Nuclear run-off experiments

To determine whether 12(S)-HETE increased αv mRNA is a result of facilitated gene transcription, in vitro transcription was performed as described (Greenberg, 1990) with some modifications. Briefly, intact nuclei were isolated from solvent (ethanol)- or 12(S)-HETE-treated CD clone 4 endothelial cells using the NP-40 lysis buffer. To initiate the transcription, nuclei were thawed and immediately added to equal amounts of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 5 mM DTT, and 1 mM of each ATP, CTP, and GTP, and 200 μCi of [α-^{32}P]UTP (760 Ci/mmol). The reaction was carried out at 30°C for 30 minutes with mixing every 5 minutes. At the end, the DNA templates were digested with RNase-free DNase I and the RNA run-off transcripts were precipitated with 10 μg/ml of tRNA, 0.2 M sodium acetate, and 2 vol. of cold ethanol at −70°C. The pellet was collected by centrifugation at 14,000 ***g*** for 1 hour and was precipitated again with 10% TCA, followed by sodium acetate and ethanol precipitation. The pellet was finally dissolved in TE buffer; 1×10^6 cpm of the labeled RNA was used to hybridize with 20 μg of immobilized cDNAs of various integrin subunits, GAPDH, or vector (pBlueScript) alone in the hybridization buffer (50% deionized formamide, 5× SSPE, 1× Denhardt's, 0.1% SDS, and 500 μg/ml tRNA) at 45°C for 48 hours. The membrane was washed under high-stringency conditions (Tang et al., 1993a). The intensity of the autoradiographic bands was determined by densitometric scanning.

Immunofluorescence, image analysis and flow cytometry

Regular immunofluorescent staining of CD clone 4 cells with antibodies against cytoskeletal proteins, integrin receptors, and endothelial cell markers was performed as described (Tang et al., 1993a-c, 1994). Immunofluorescence imaging analysis of cell surface integrin αv or αvβ3 was performed as detailed previously (Tang et al., 1993c). Briefly, CD clone 4 endothelial cells were treated with 0.1 μM of 12(S)-HETE for 1, 2, 4, 8, 16, and 24 hours, after which cells were fixed with 3.7% paraformaldehyde in PBS (containing 1 mM of Ca^{2+} and Mg^{2+}, and 5% sucrose, pH 7.4) for 15 minutes. Following washing with PBS, cells were incubated with 20% normal goat whole serum in 4% BSA-PBS at 37°C for 30 minutes to block the non-specific binding. Cells were then incubated with either mAb to αv or rabbit pAb to αvβ3 (20 μg/ml) and subsequently with FITC-conjugated goat anti-mouse or anti-rabbit IgG (1:200) for 45 minutes at 37°C. MOPC ascites (IgG) or non-immune rabbit IgG was used as antibody control. Fluorescence image analysis with a large population of CD clone 4 cells was performed on the ACAS 470 argon laser fluorescence imaging cytometer (Tang et al., 1993c). The FITC label was excited at 488 nm and the fluorescence emission measured at 530 nm. The relative fluorescence intensity was indicated by the color of the images, ranging from green (low fluorescence) to red (high fluorescence), and the data were analyzed as described before (Tang et al., 1993c). The flow cytometric labeling of integrin receptors was performed as described previously (Tang et al., 1993b,c, 1994). Briefly, CD clone 4 cells treated with 0.1 μM of 12(S)-HETE or PMA were dissociated with 0.2 mM ETDA, together with gentle pipetting. After washing with DMEM, cells were fixed with 3.7% paraformaldehyde in PBS. Then cells were sequentially incubated with 20% goat whole serum, primary antibodies (i.e. 20 μg/ml of anti-αvβ3, αvβ5, or α5β1, or Rb IgG control), and FITC-labeled secondary antibodies (1:500). Finally cell surface fluorescence for individual integrin receptors was analyzed on a Epics Profile II flow cytometer (Coulter, Hialeah, FL).

Adhesion assays

Adhesion of CD clone 4 cells to vitronectin was performed as previously described (Tang et al., 1993b,c). Twenty-five × 10^3 CD clone 4 cells treated with 12(S)-HETE, PMA (0.1 μM, 4 hours) or ethanol were seeded onto vitronectin (1-5 μg/ml) immobilized on 96-well flat-bottom culture plates. The adhesion assays were performed for 45 minutes in the presence of mAbs to αv, α5 or αvβ5 or pAb to αvβ3 or α5β1 (30 μg/ml), or corresponding control antibodies MOPC and rabbit IgG, respectively. The adhesion was quantitated as previously described (Honn et al., 1989; Tang et al., 1993c).

RESULTS

Our previous series of studies demonstrated that 12(S)-HETE modulates the αvβ3 expression in CD clone 3 microvascular endothelial cells in a post-transcriptional, PKC- and cytoskeleton-dependent manner (Tang et al., 1993c, 1994). In an attempt to determine whether this 12(S)-HETE effect is restricted to CD clone 3 cells or is, rather, a general phenomenon, we screened by quantitative RT-PCR several additional endothelial cell populations, which included CD clone 4 murine vascular endothelial cells, rat and mouse aorta, and mouse kidney and liver endothelial cells, for mRNA levels of αv, β3, α5, and β1. In most of these endothelial cell lines examined, we did not observe consistent alterations in the mRNA levels of these integrin subunits following 12(S)-HETE stimulation. However, to our surprise, the message levels of αv in CD clone 4 cells appeared to be increased by 12(S)-HETE (see below). Since both CD clone 3 and clone 4 endothelial cells were prepared from mouse pulmonary microvasculature, the contrasting effects of 12(S)-HETE on αvβ3 in these two cell clones appeared to indicate cellular heterogeneity. CD clone 3 cells have been well characterized and extensively used in our previous experiments (Chopra et al., 1990; Tang et al., 1993b,c, 1994). At the time we performed the RT-PCR screening, CD clone 4 cells were characterized at the morphological level and were considered to be endothelia, based on their overall similarity (morphology, growth properties, etc) to CD clone 3 cells.

The differential responses of these two cell lines to 12(S)-HETE in regard to the αvβ3 expression thus prompted us to further evaluate the cellular identity of CD clone 4 cells. As shown in Fig. 1, CD clone 4 cells cultured in DMEM supplemented with 10% serum and 0.05% gelatin overall resembled CD clone 3 cells (Tang et al., 1993b,c) except that some cells were more elongated than CD clone 3 cells and that confluent CD clone 4 cell cultures did not demonstrate a typical 'cobblestone' morphology as did CD clone 3 cells (Fig. 1A; data not shown). CD clone 4 cells, like CD clone 3 cells, expressed vimentin (Fig. 1B), but did not express cytokeratin (Fig. 1C), desmin (Fig. 1D), and smooth muscle cell specific α-actin (data not shown). The negative staining of CD clone 4 cells with these molecules was not due to a lack of reactivity of the antibodies, since mAb to desmin stained murine 3T3 fibroblasts (Fig. 1E) and mAb to cytokeratin stained murine B16a melanoma cells (data not shown). CD clone 4 cells expressed integrin αvβ3 (Fig. 1F, arrowheads) and α5β1 (Fig. 1G, arrowheads), but these integrin receptors were not localized to focal adhesions as distinctly as in CD clone 3 cells (Tang et al., 1993c). CD clone 4 cells also expressed P-selectin (Fig. 1H), which was stored in intracellular granules (McEver, 1991), and PECAM-1, which was localized to typical cell-cell junctions (Fig. 1I, arrowheads). Finally, staining of CD clone 4 cells with several antibodies (Table 1) against von Willebrand factor revealed a strong labeling of Weibel-Palade bodies (Fig. 1J). Taken together, the above experimental data demonstrate that CD clone 4 cells are endothelial cells.

The above experimental results suggested that it would be interesting to further analyze the differential effects of 12(S)-HETE on the αvβ3 expression in CD clone 3 and clone 4 cells. As the first approach to this end, quantitative RT-PCR was performed. For comparison, RT-PCR analyses of α5β1 were simultaneously performed. Two strategies were adopted in order to better quantitate the mRNA levels. In the first, parallel RT-PCR, RT-PCR/Southern hybridizations were performed

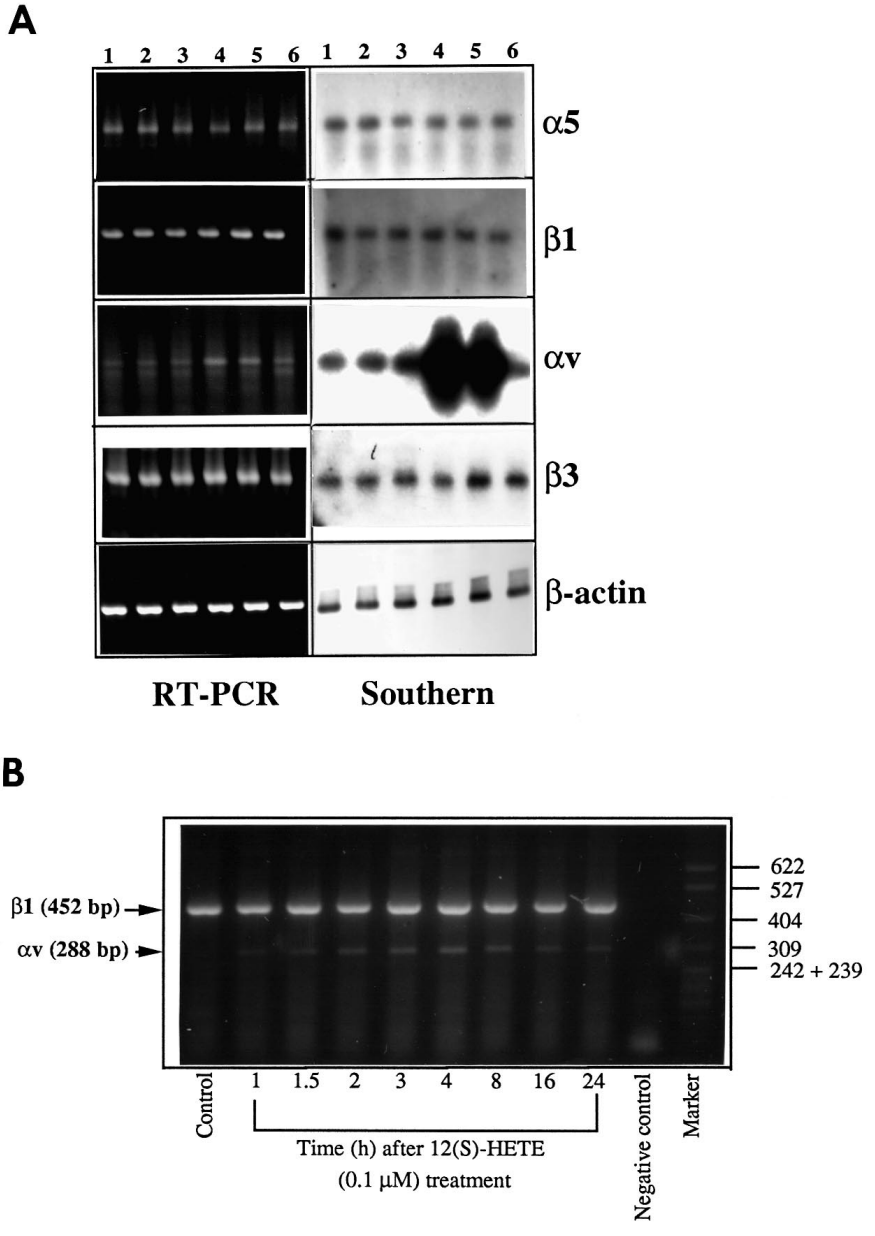


Fig. 2. 12(S)-HETE increases the mRNA levels of integrin αv of CD clone 4 cells as assessed by quantitative RT-PCR. (A) Parallel RT-PCR analysis using β-actin as the external control. Cultured confluent CD clone 4 endothelial cells were treated with either solvent (ethanol; 4 hours, lane 1) or 0.1 μM of 12(S)-HETE for 1, 2, 4, 8, and 24 hours (lanes 2-6) and total RNA was isolated and used in quantitative RT/PCR (left panel). Preliminary experiments were performed to set up conditions to ensure exponential amplification (see Materials and Methods) and the detailed cycling profiles for each individual PCR are given in Table 2. After PCR, 20 μl of product was run in 1% agarose gel and transferred to nylon membrane, which was hybridizd to various fluorescein-labeled cDNA probes (indicated on the right) and the bands detected using the ECL system (right panel). (B) One-tube RT-PCR. CD clone 4 cells were treated with either ethanol control (4 hours) or 0.1 μM 12(S)-HETE for the time intervals indicated. The RT and PCR for αv and β1 were performed in the same tube using conditions that ensure the exponential amplification for both integrin subunits (see Materials and Methods). Negative control was run without adding RNA to the RT reaction. The molecular markers are shown on the right. The normalized (against the β1 band) values from densitometric scanning are 1.0 (control), 2.4 (1 hour), 3.6 (1.5 hours), 5.6 (2 hours), 7.2 (3 hours), 9.5 (4 hours), 7.7 (8 hours), 5.3 (16 hours), and 3.0 (24 hours), respectively.

with each individual integrin subunit and with β-actin. As described in Materials and Methods, pilot experiments were set up to establish the optimal cycling profiles (Table 2), which gave exponential amplification for each molecule. Then the mRNA levels of integrin subunits were normalized against that of β-actin. As shown in Fig. 2A, 12(S)-HETE increased the mRNA level of αv (>20 fold on Southern blot shown in Fig. 2A) in a time-dependent manner. The enhancement was observed at ~1 hour and peaked about 4 hours after treatment (Fig. 2A). Repeat experiments consistently demonstrated the 12(S)-HETE-enhanced αv mRNA. In some experiments, the mRNA level of β3 also appeared to be elevated following 12(S)-HETE stimulation, but the increase was much less dramatic and consistent than that of αv. In contrast, no significant and consistent alterations in the levels of α5 and β1 were observed (Fig. 2A). In order to minimize the experimental errors derived from performance inaccuracies in the parallel RT-PCR, we developed a one-tube RT-PCR protocol in which the RT reaction and PCR for αv and β1 were performed in the same tube using the cycling conditions that ensured the exponential amplification for both pairs of primers (see Materials and Methods). As shown in Fig. 2B, the basal mRNA level of β1 was much higher than that of αv. 12(S)-HETE treatment resulted in a time-dependent increase in the αv mRNA level, which peaked at ~4 hours (>10-fold by scanning the negative) although the β1 mRNA level did not change (Fig. 2B). Southern hybridization confirmed the RT-PCR results (data not shown). A similar one-tube RT-PCR was set up to co-amplify the α5 (323 bp) and β3 (521 bp) and, as expected, no significant changes were noted. These data led us to focus most of the subsequent studies on αv.

To confirm the 12(S)-HETE effect on the induction of αv mRNA, a sensitive RNase protection assay was performed using ^{32}P-labeled riboprobes. As shown in Fig. 3, 12(S)-HETE treatment increased αv mRNA most significantly at ~4 hours (~3-fold increase as determined by desentometric scanning) after stimulation and persisted up to 16 hours. The effect declined at 24 hours after treatment (Fig. 3) and dropped to the control level by 48 hours (data not shown). No effects of 12(S)-HETE were observed on other integrin subunits examined. Alternatively, a solution hybridization method was adopted to quantitate the αv mRNA of CD clone 4 cells following 12(S)-HETE stimulation. Using this approach, multiple samples could be measured simultaneously. As presented in Table 3 and Fig. 4, 12(S)-HETE time-dependently increased the mRNA levels of integrin αv, with the maximal effect (nearly 3-fold increase) observed again at ~4 hours post-stimulation. As observed with RT-PCR and RNase protection assays, 12(S)-HETE-induced αv mRNA expression declined by 24 hours after stimulation. The 12(S)-HETE enhanced αv message was also confirmed by northern blotting, which demonstrated that 12(S)-HETE, in a dose-dependent manner, increased the mRNA levels of αv (Fig. 5). The induction was noted at a 12(S)-HETE concentration of 0.01 μM and peaked at 0.1 μM where a greater than 2-fold increase was found (Fig. 5). Interestingly, at the highest dose (i.e. 1.0 μM) of 12(S)-HETE examined, the level of αv mRNA decreased slightly below the control level (Fig. 5).

To explore the mechanism of the 12(S)-HETE effects, CD clone 4 cells were pretreated with either several protein kinase inhibitors or cycloheximide before 12(S)-HETE stimulation.

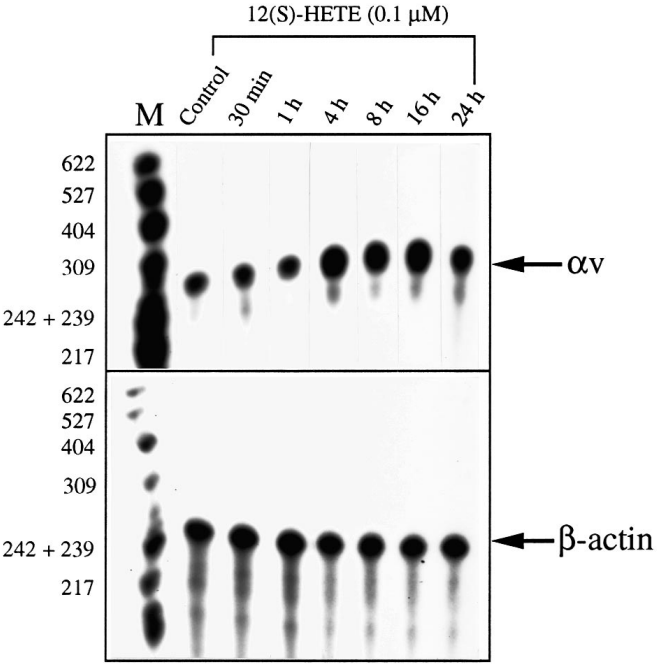


Fig. 3. Time course of the induction of integrin αv by 12(S)-HETE as measured by ribonuclease protection assays. Confluent CD clone 4 cells were treated with either ethanol (4 hours; control) or 0.1 μM 12(S)-HETE for various time points as indicated. An 8 μg sample of total RNA was used in the protection assays and the expected, protected fragment (~250 bp; see Materials and Methods, and Table 3 for details) was identified by autoradiography. A β-actin fragment of similar size was used as the control. The molecular mass markers (in bp) shown on the left were pBR322/*Msp*I fragments labeled with ^{32}P. Note that protection assays for αv and β-actin were run separately due to the similar sizes of the protected fragments.

Table 3. Induction of integrin αv mRNA by 12(S)-HETE

	0	1 h	2 h	4 h	8 h	24 h
Control	8261±840	11 928±1 360	12 450±1 010	9 205±346	11 815±1 029	9 327±148
12(S)-HETE		15 392±1 806	28 820±3 660	25 890±1 680	31 490±2 775	12 840±535

CD clone 4 cells were treated with either ethanol (control) or 12(S)-HETE (0.1 μM) in serum-free DMEM for 5 minutes and then cultured in serum-containing DMEM for the time intervals indicated. At the end of treatment, cells were rinsed (3×) with serum-free DMEM and total RNA isolated from each sample. A 10 μg sample of total RNA was hybridized with ^{35}S-labeled antisense probes of either integrin αv (1 million cpm/sample) or β-actin (250,000 cpm/sample) at 42°C for 24 hours (see Materials and Methods for details). Following RNase digestion, protected fragments were dissolved in 30 μl of DEPC water and a 10 μl sample was collected onto glass fiber filter discs, which were washed with 10% TCA, 3 times. Dried filter discs were then counted in a β-counter with scintillation fluid.

The cpm values obtained with αv were normalized to corresponding β-actin cpm values for both control and 12(S)-HETE treated groups. Each condition was run in triplicate. Shown are the mean ± s.d. The cpm value for αv sense probe hybridization in this experiment was 230±20.

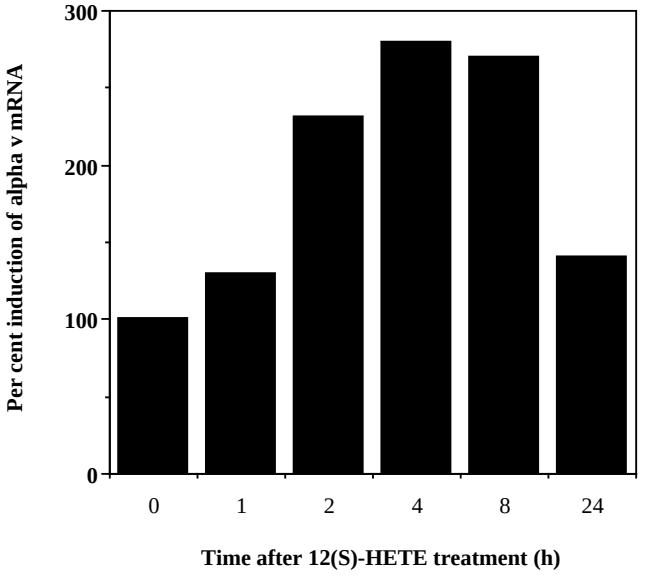


Fig. 4. Induction of integrin αv mRNA by 12(S)-HETE as revealed by solution hybridization. Shown is the percentage increase in the αv mRNA levels derived from data in Table 3.

RNase protection assays were then performed to quantitate the mRNA levels of αv. As shown in Fig. 6A, 12(S)-HETE (0.1 μM; 4 hours) stimulation resulted in an upregulation (3.4-fold increase for lane 2 and 3.6-fold increase for lane 6) in the level of αv mRNA. The 12(S)-HETE effect was inhibited (75% inhibition) by pretreating CD4 cells with specific PKC inhibitor calphostin C (0.5 μM; 15 minutes) but not by PKA inhibitor H8 (5 μM; 15 minutes). Genistein (50 μg/ml; 15 minutes), a general protein tyrosine kinase inhibitor, in most cases did not inhibit the stimulatory effect of 12(S)-HETE (Fig. 6A). To examine whether 12(S)-HETE-upregulated αv mRNA was dependent on protein synthesis, CD4 endothelial cells were pretreated with cycloheximide (50 μM; 1 hour) followed by 12(S)-HETE stimulation (0.1 μM; 4 hours) in the presence of the inhibitor. No effect of cycloheximide on the 12(S)-HETE-induced αv mRNA was observed (Fig. 6A). The above experimental data suggest that 12(S)-HETE-upregulated mRNA levels of integrin αv depend on PKC activation but not on ongoing protein synthesis. A support for the involvement of PKC in the 12(S)-HETE effect was that PMA, a well-known PKC activator, also time-dependently increased the αv mRNA levels at 100 nM (Fig. 7). Like 12(S)-HETE, PMA-stimulated αv mRNA expression was observed at 1 hour, plateaued at 4 hours, and declined at 24 hours (but not to the basal level) post-stimulation (Fig. 7). Next, studies were undertaken to determine whether 12(S)-HETE treatment alters the stability of the αv mRNA. CD clone 4 cells were first treated with solvent or 12(S)-HETE (0.1 μM) for 4 hours, then rinsed to remove 12(S)-HETE, and then followed with 5 μg/ml of actinomycin D treatment for various time periods up to 6 hours. RNA was subsequently isolated for the protection assays and the ratio of the αv mRNA levels (between 12(S)-HETE-treated groups and the control) as a function of time represented a measurement of the relative stability of αv mRNA. Preliminary results from such measurements did not reveal a significant change in the ratio of αv mRNA levels after blocking ongoing RNA synthesis by actinomycin D (Fig. 6B), suggesting that 12(S)-

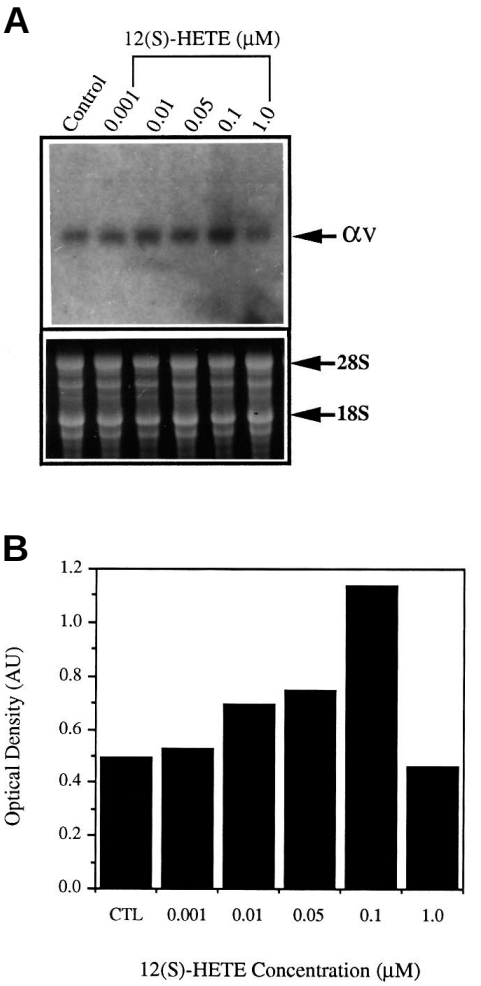


Fig. 5. Dose study of the induction of αv mRNA by 12(S)-HETE as determined by northern blotting. (A) A 10 μg sample of mRNA prepared from CD clone 4 cells treated with either ethanol or increasing doses of 12(S)-HETE for 4 hours (37°C) was separated by formaldehyde/agarose gel, transferred to GeneScreen Plus nylon membrane and probed with ^{32}P-labeled αv cDNA probe. The expected αv band (~7.0 kb) is indicated by an arrow. Shown is an autoradiograph exposed for 10 days with an enhancing screen at –80°C. The ethidium bromide-stained picture (lower panel) was shown to demonstrate euqal loading of samples. Note that this blot was also reprobed with β-actin (data not shown). (B) Densitometric scanning results of the bands presented in A.

HETE may not alter the half-life of αv mRNA. In fact, in the experiment presented in Fig. 6B the ratios for 90, 180, and 360 minutes (of actinomycin treatment) were 1.7, 1.8, and 1.1, respecitvely. The reason for the decreased ratio at the later time point of actinomycin D treatment is unknown. In another set of experiments in which CD clone 4 cells were first treated with actinomycin D (5 μg/ml, 4 hours) followed by 12(S)-HETE stimulation in the presence of the inhibitor, no induction of αv mRNA by 12(S)-HETE was observed, thus implicating transcriptional involvement in the 12(S)-HETE effect.

Nuclear run-off experiments were subsequently performed to determine whether 12(S)-HETE-induced increase in αv mRNA results from an upregulated gene transcription. As shown in Fig. 8, the basal level of transcriptional activity for integrin α5 was easily detectable and 12(S)-HETE treatment

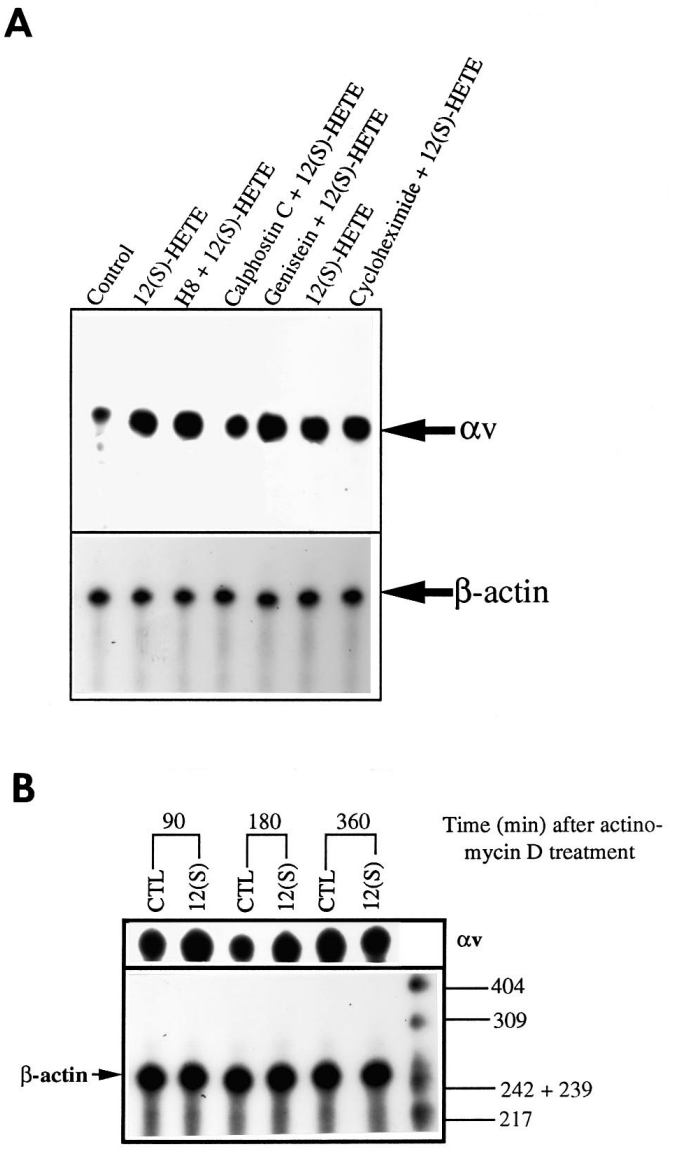


Fig. 6. Exploration of the mechanisms of the 12(S)-HETE induction of CD clone 4 cell integrin αv. (A) Effects of protein kinase and protein synthesis inhibitors on 12(S)-HETE stimulation of integrin αv message. CD clone 4 cell cultures were pretreated with various inhibitors (see text for details) before stimulated with 0.1 μM 12(S)-HETE for 4 hours. A 5 μg sample of total RNA was used in the RNase protection assays as described for Fig. 3. (B) 12(S)-HETE does not appear to affect the half-life of αv mRNA. CD clone 4 endothelial cells were first treated with 12(S)-HETE (0.1 μM) or solvent for 4 hours followed by washing away of the eicosanoid. Subsequently cells were exposed to 5 μg/ml actinomycin D for the time periods indicated. A 15 μg sample of total RNA was used in the protection assays. After normalization of the αv mRNA levels against those of β-actin, the ratio of the αv mRNA levels between 12(S)-HETE-treated conditions and controls was determined. In this experiment the ratios for 90, 180, and 360 minutes were 1.7, 1.8 and 1.1, respecitvely.

resulted in only a marginal increase in the transcription. In contrast, the basal level of transcription for integrin αv was extremely low (the band was detecatable only after extended exposure) and 12(S)-HETE treatment led to a significant increase in the transcriptional activity of αv gene (Fig. 8).

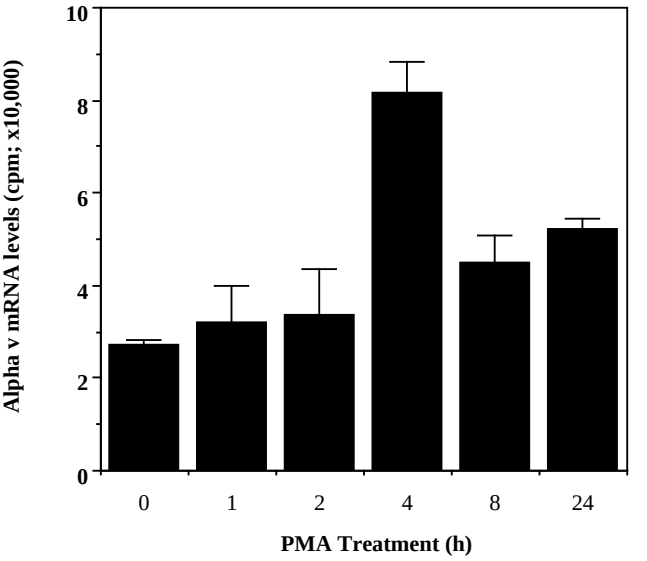


Fig. 7. PKC activator PMA induces the αv mRNA in CD clone 4 endothelial cells. Cultured CD clone 4 cells were treated with PMA (0.1 μM) for the time periods indicated, total RNA was isolated, and then solution hybridization (10 μg total RNA) performed was using [^{35}S]labeled antisense αv (1×10^6 cpm/sample) or β-actin (250,000 cpm/sample) riboprobes, as detailed in Materials and Methods. The cpm values of αv hybridization were normalized to corresponding β-actin hybridization values. Triplicate samples were performed for each time point for both αv and β-actin. The results were expressed as the mean cpm ± s.d.

The above data collectively suggest that 12(S)-HETE activates αv gene transcription, resulting in an increase in the mRNA content of this integrin subunit. Immunofluorescence imaging analysis, flow cytometry and cell adhesion assays were next performed to determine whether there is any correlation between increased αv message and protein expression on the cell surface. For the digitized immunofluorescence imaging analysis (Tang et al., 1993c), CD clone 4 cell cultures at 70-90% confluence were surface labeled with either mAb to αv (data not shown) or rabbit pAb (polyclonal antibody) to αvβ3 (Fig. 9). These two antibodies were previously shown to immunostain and immunoprecipitate corresponding integrins from the CD clone 3 cells (Tang et al., 1993c, 1994). CD clone 4 microvascular endothelial cells under unstimulated conditions expressed very low amount of αvβ3 integrin on their surface (Fig. 9A). 12(S)-HETE stimulation resulted in a time-dependent increase in the surface expression of αvβ3, with a maximum induction at ~4 hours (Fig. 9D). By 24 hours post 12(S)-HETE treatment, a significant amount of αvβ3 above the control level could still be detected (Fig. 9F). Flow cytometric analysis (Fig. 10) also revealed a low basal level expression (Fig. 10A) of integrin αvβ3 on CD clone 4 cell surfaces, which was enhanced by stimulation with 12(S)-HETE (Fig. 10B) as well as PMA (Fig. 10C). In contrast, 12(S)-HETE and PMA did not significantly affect the surface expression of α5β1 (Fig. 10D-F). CD clone 4 cells also expressed a very low level of αvβ5 on the surface (Fig. 10G). Both 12(S)-HETE and PMA appeared to upregulate αvβ5 surface expression (Fig. 10H and I), but to a much lesser extent compared to the induction of αvβ3. 12(S)-HETE and PMA also enhanced CD clone 4 cell adhesion to vitronectin (Fig. 11). By 4 hours, adhesion of CD

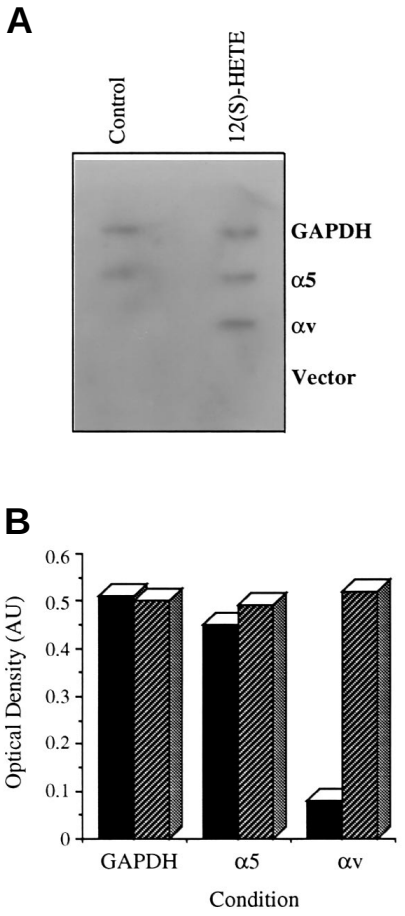


Fig. 8. 12(S)-HETE activates the gene transcription of integrin αv. (A) Nuclear run-off experiments. Confluent CD clone 4 endothelial cells were treated with either solvent (control) or 12(S)-HETE (0.1 μM) for 4 hours and then cells were harvested, intact nuclei were prepared with the NP-40 lysis buffer, and nuclear run-off experiments performed (see Materials and Methods). The picture was from a 3-day exposure autoradiogram and longer exposures revealed a faint band of αv in the control (data not shown). Vector, pBlueScript.
(B) Scanning results of data from A.

clone 4 cells to vitronectin increased by >2-fold, which was inhibited by either mAb to αv or pAb to αvβ3, but not by anti-α5β1 or -αvβ5, or nonimmune RbIgG (Fig. 11). Anti-αvβ3 also appeared to inhibit basal adhesion (Fig. 11B). PMA demonstrated a stronger upregulatory effect on CD clone 4 cell adhesion, which was also inhibited by anti-αvβ3, but not by anti-α5β1 or -αvβ5, or nonimmune RbIgG (Fig. 11B).

The above experimental data indicate that in CD clone 4 cells the basal transcriptional activity of the αv gene and the basal levels of αv mRNA are extremely low (see Fig. 2 and Fig. 8). RNA derived from both CD clone 3 and clone 4 cells was used in RT-PCR/Southern blotting to determine whether there is any difference in the basal level expression of αv mRNA in both cell lines (Fig. 12). For comparison, RT-PCR for PECAM-1 also was performed before and after 12(S)-HETE stimulation (Fig. 12) using primers and cycling conditions previously described (Tang et al., 1993a). Both CD clone 3 and clone 4 cells expressed comparable basal levels of mRNA for PECAM-1, which were not altered by 12(S)-HETE stimulation (Fig. 12). In sharp contrast, CD clone 4 cells, under the unstimulated conditions, expressed much lower mRNA levels for integrin αv than do CD clone 3 cells (Fig. 12). As observed before, 12(S)-HETE treatment (0.1 μM, 4 hours) increased the mRNA levels of integrin αv in CD clone 4 cells but not in CD clone 3 cells (data not shown; also refer to Tang et al., 1993c, 1994).

DISCUSSION

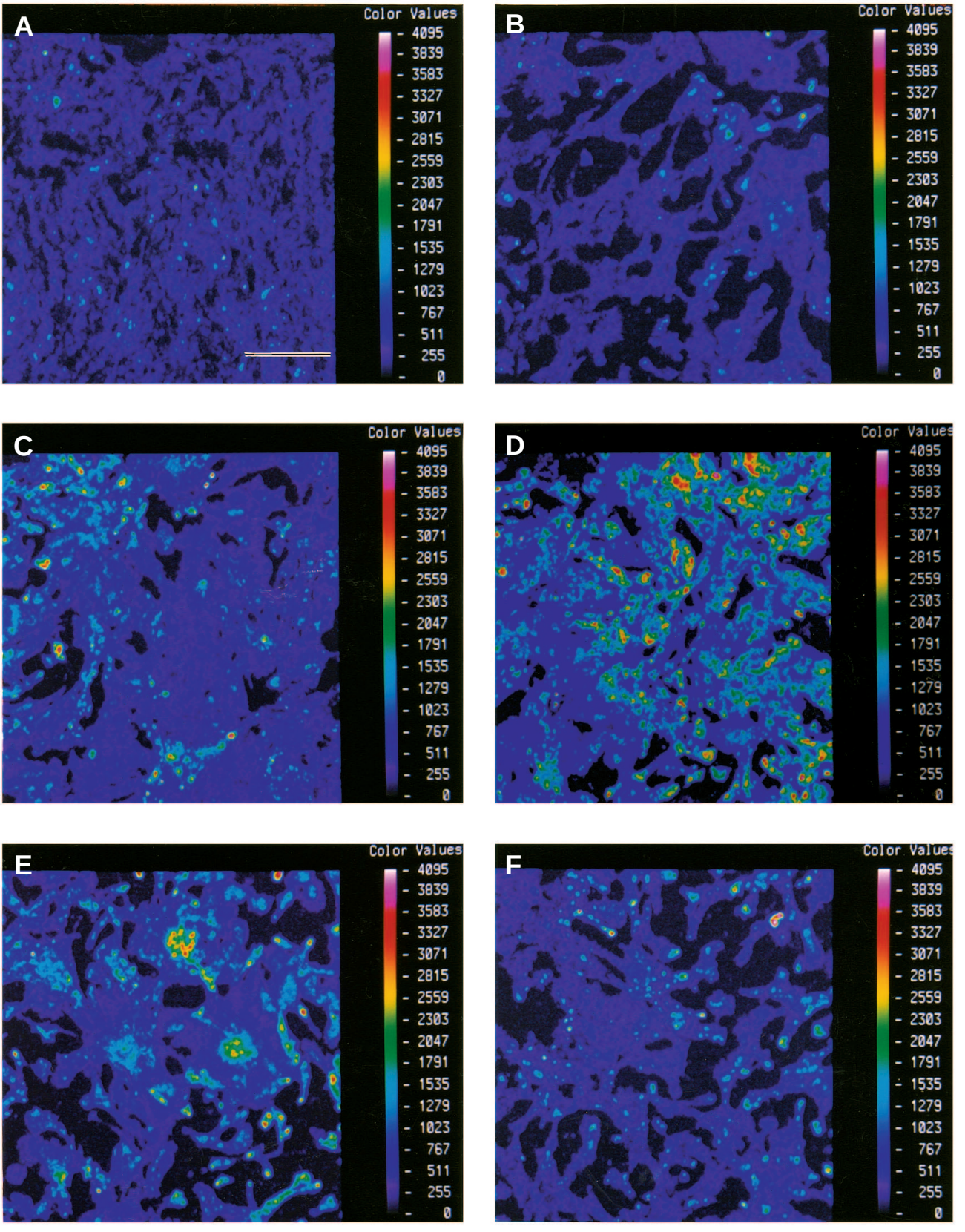
The functional activity of integrin receptors is strictly regulated. Two major mechanisms exist to regulate the function of integrins, i.e. qualitative and quantitative (Hynes, 1992). Qualitative activation involves conformational changes (due to agonist stimulation and/or ligand binding) in integrin receptors without significant alterations in the level of expression, which has been observed with β1, β2, and β3 subfamily integrins (Chan et al., 1991; Hynes, 1992; Wright and Meyer, 1986). Quantitative regulation of integrin function is by modulating the level of their expression, exemplified by rapid mobilization of the αIIbβ3 integrin receptors from the intracellular pool to the plasma membrane of platelets in response to thrombin stimulation (Wencel-Drake et al., 1986) or in tumor cells in response to 12(S)-HETE (Timar et al., 1995). For any individual integrin heterodimer, both mechanisms may operate to fine tune its biological activity.

αvβ3 is one of the major integrins expressed by vascular endothelial cells. The receptor expressed at the cell-matrix contact sites (i.e. the focal adhesions) and intercellular junction zones plays an essential role in mediating endothelial cell adhesion to matrix and maintaining the integrity of endothelial cell monolayers. Compared to α5β1, which mediates cell adhesion almost exclusively to fibronectin, integrin αvβ3 mediates interactions of endothelial cells with a wide spectrum of ligands. Not surprisingly, αvβ3 in endothelial cells appears to be much more dynamic and functionally more versatile and important than α5β1. The expression/function of αvβ3 has been shown to be modulated by, in addition to divalent cations and several integrin-associated proteins (Bartfeld et al., 1993; Schwartz et al., 1993; Smith et al., 1994), a large array of bioactive mediators. These include TGF-β (Ignotz et al., 1989), tumor necrosis factor α and interferon γ (Defilippi et al., 1991), interleukin-1 (Lafrenie et al., 1992), GM- and M-CSF (colony-stimulating factor; De Nichilo and Burns, 1993), 1α,25-dihydroxyvitamin D_3 (Medhora et al., 1993), prostaglandin E_2 (Milam et al., 1991), basic fibroblast growth factor (Klein et al., 1993), eicosanoid 13-HODE (13-hydroxyoctadecaenoic acid; Buchanan et al., 1993), and phospholipids (Conforti et

Fig. 9. Immunofluorescence imaging analysis demonstrating that 12(S)-HETE induces a time-dependent increase in the surface expression of integrin αvβ3. CD clone 4 endothelial cells were treated with solvent (4 hours; A) or 0.1 μM 12(S)-HETE for 1 hour (B), 2 hours (C), 4 hours (D), 8 hours (E), or 24 hours (F) and then processed for immunofluorescent staining with rabbit pAb to αvβ3 and FITC-labeled goat anti-rabbit IgG. ACAS digitized imaging analysis was performed as previously described (Tang et al., 1993c) and the level of immunofluorescent label is represented by color-coded values. Similar results were obtained with a mAb to αv (data not shown). Bar, 10 μm.

al., 1990). Many of these modulators such as TGF-β and interleukin-1 alter the mRNA levels of αv and/or β3.

12(S)-HETE is a lipoxygenase-derived hydroxy fatty acid possessing a wide spectrum of biological activities including regulation of integrin expression, tumor cell adhesion to endothelial cells and matrix, and tumor metastasis (reviewed by Honn et al., 1994). Previous studies demonstrated that this eicosanoid upregulates the surface expression of αvβ3 in CD



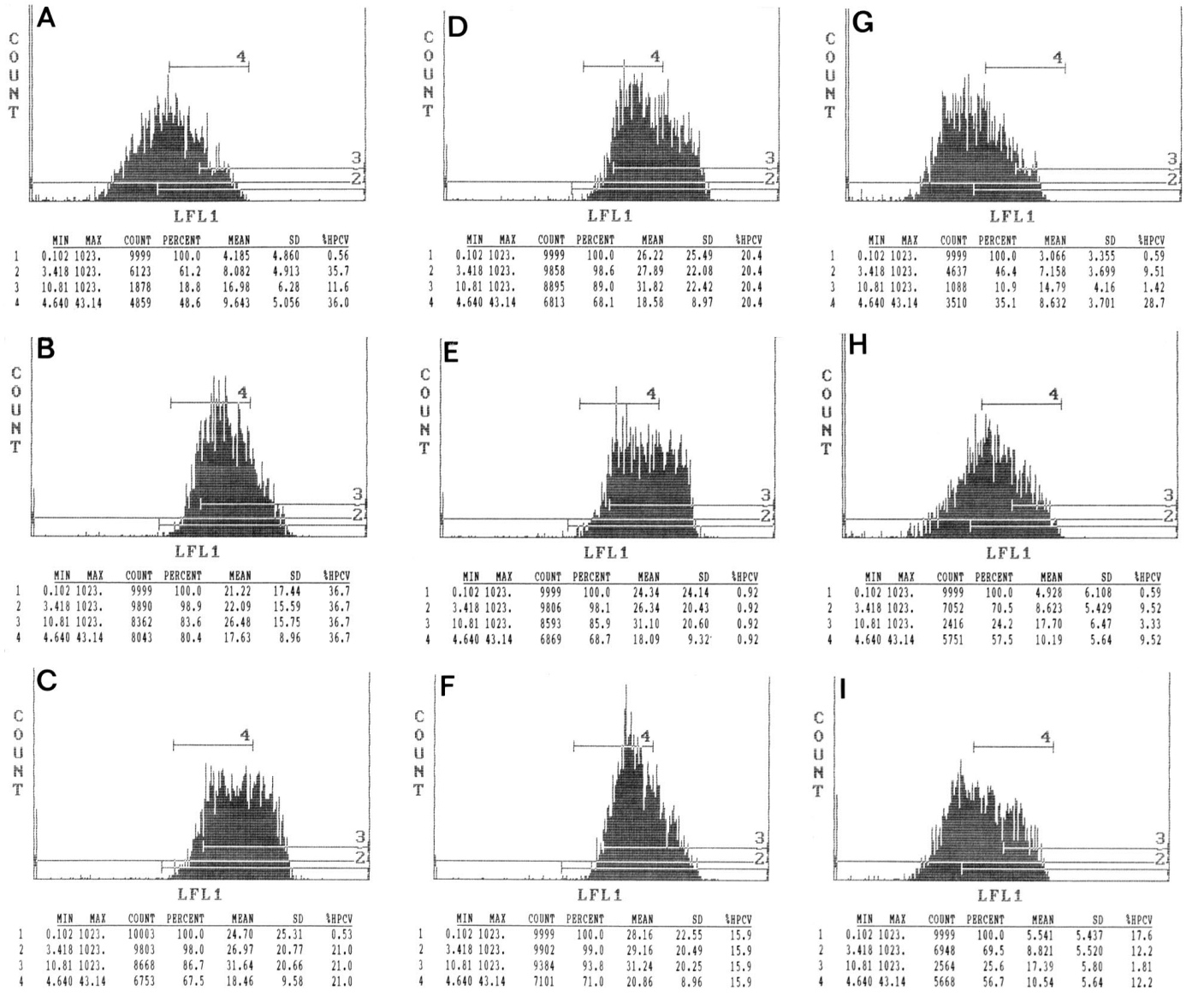


Fig. 10. 12(S)-HETE and PMA promote the surface expression of integrin αvβ3 on CD clone 4 cells as revealed by flow cytometric analysis. Adherent CD clone 4 cells stimulated with 0.1 μM 12(S)-HETE (B, E, and H) or PMA (C, F, and I), or ethanol control (A, D, and G) for 4 hours were surface labeled with RbIgG (control antibody), polyclonal anti-αvβ3 (A-C) or anti-α5β1 (D-F), or monoclonal anti-αvβ5 (P1F6; G-I) all at 20 μg/ml and the fluorescent (FITC) label was analyzed on a Epics Profile II flow cytometer. Shown under each histogram are both % positive labeling and mean fluoresence. RbIgG labeling resulted in negligible fluorescence.

clone 3 endothelial cells (Tang et al., 1993c, 1994). However, 12(S)-HETE treatment did not alter the mRNA levels of αv or β3 (as revealed by northern blotting) but rather, in a PKC- and cytoskeleton-dependent manner, rapidly mobilized αvβ3 molecules from the cytosolic pool to the plasma membrane of CD clone 3 endothelial cells (Tang et al., 1993c, 1994). In the current study, we demonstrated that 12(S)-HETE also appears to modulate the integrin expression at the gene level. When screening by RT-PCR/Southern blotting for the 12(S)-HETE effects on integrin expression in several tissue-specific endothelial cell populations, we consistently found that 12(S)-HETE upregulated the mRNA levels of integrin αv in CD clone 4 endothelial cells. This was surprising, since both CD clone 3 and clone 4 endothelial cells were similarly prepared from mouse pulmonary tissue and the 12(S)-HETE effect on CD clone 3 cell integrin expression was shown to be a post-transcriptional process (Tang et al., 1994). Therefore, it became necessary to further characterize CD clone 4 cells with a large panel of antibodies directed against cytoskelatal proteins, adhesion molecules, and endothelial cell markers. The results demonstrate that CD clone 4 cells are true endothelial cells and not mesenchymal elements such as fibroblasts or smooth muscle cells.

The upregulation of αv message by 12(S)-HETE in CD clone 4 cells was revealed by two different RT-PCR protocols (i.e. parallel and one-tube RT-PCR) in conjunction with Southern blotting with subunit-specific cDNA probes. The 12(S)-HETE effect was subsequently confirmed by ribonuclease protection assays, solution hybridization and northern blotting. 12(S)-HETE induction of αv mRNA occurs very

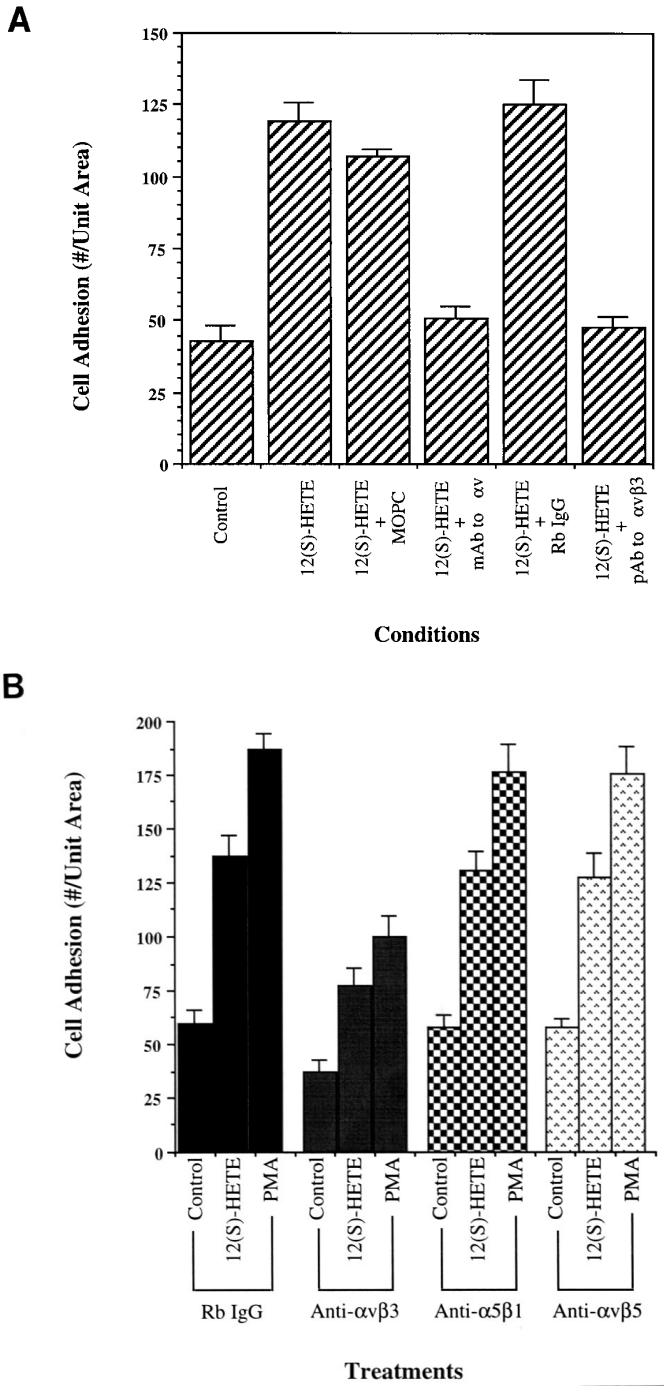


Fig. 11. (A) 12(S)-HETE increases CD clone 4 cell adhesion to vitronectin. CD clone 4 endothelial cells were treated with ethanol control or 0.1 μM 12(S)-HETE for 4 hours. Then cells were dissociated with 2 mM EDTA together with gentle pipetting. After washing, cell adhesion assay was performed with 2.5×10^4 cells/well in 96-well flat-bottom culture plates on which vitronectin (1 μg/ml) had been immobilized. In some conditions, MOPC or mAb to αv (10 μg/ml), or rabbit IgG or rabbit pAb to αvβ3 (30 μg/ml) was included in the adhesion mixtures. The adhesion proceeded for 45 minutes, after which nonadherent cells were removed by gently washing the plates 4 times. Quantitation of adhesion was done by counting the adherent cells in a distinct unit of microscopic area (Honn et al., 1989). Each condition was run in quadriplicate and data presented as the mean ± s.d. (B) Similar cell adhesion to vitronectin (5 μg/ml) was performed for 30 minutes with CD clone 4 cells stimulated with either 12(S)-HETE or PMA (both at 0.1 μM for 4 hours). The adhesion assays were performed in the presence of RbIgG, rabbit polyclonal anti-αvβ3 or -α5β1, or monoclonal anti-αvβ5 (all at 20 μg/ml). Each condition was run in triplicate and results presented as mean ± s.d.

rapidly, i.e. within 1-2 hours, reaches the peak level at ~4 hours, and gradually declines by 24 hours. The 12(S)-HETE effect is insensitive to cycloheximide treatment, suggesting the independence of protein synthesis. 12(S)-HETE does not appear to alter the half-life of the αv mRNA, since blocking the ongoing RNA synthesis with actinomycin D post-12(S)-HETE treatment does not reveal an increased ratio of the αv mRNA between the treated samples and the controls. However, pretreatment of CD clone 4 cells by actinomycin D followed by 12(S)-HETE stimulation prevented the 12(S)-HETE induction of αv mRNA, suggesting a transcriptional involvement. Subsequent nuclear run-off experiments confirmed that 12(S)-HETE activates the gene transcription of integrin αv but not α5.

The mechanism underlying the contrasting responses of two separately cloned pulmonary microvascular endothelial cell populations to 12(S)-HETE stimulation is unclear. It is known that vascular endothelial cells of different vasculatures (e.g. large vs small vessels) from different tissue and organ sites demonstrate heterogeneity in respect to various morphological, biochemical, and functional properties (Gerritsen, 1987; Kumar et al., 1987; Madri and Williams, 1983; Page et al., 1992). Furthermore, endothelial cells derived from the same microvasculature may also demonstrate fundamental regional differences which are maintained in vitro and modulated by culture conditions (Kumar et al., 1987; Fajorrdo, 1989; Zetter, 1988). For example, Rupnick et al. (1988) established four endothelial cell populations from mouse brain microvessels and they found that these different populations demonstrated distinct phenotypic properties in culture, which might represent different vessel types such as capillaries, venules, or arterioles. Also, in vitro culture conditions and matrix components have been shown to modulate the phenotypic properties of microvascular endothelial cells (Madri and Williams, 1983). Although both CD clone 3 and clone 4 cells are isolated from mouse pulmonary microvasculature, the precise microanatomical source from which these endothelial cells are derived remains unknown. In fact, CD clone 3 and CD clone 4 cells exhibit different characteristics with regard to morphology and integrin expression. Morphologically, CD clone 4 cells are generally more elongated and at confluency do not exhibit a typical ‘cobblestone’ morphology like that of CD clone 3 cultures. RT-PCR/Southern analysis of αv mRNA consistently reveals that CD clone 3 cells have a much higher basal level of αv message than clone 4 cells, although both cell lines possess comparable message levels of PECAM-1, whose mRNA expression is not affected by 12(S)-HETE treatment (Fig. 12; also see Fig. 2). Interestingly, the basal level of αv mRNA in CD clone 4 cells is extremely low compared with the mRNA levels of other integrin subunits such as β3, α5, and β1 (see Fig. 2), which may partly explain the gene-specific effect of 12(S)-HETE on αv (see below). The differential expression of basal level of αv mRNA in CD clone 3 and clone

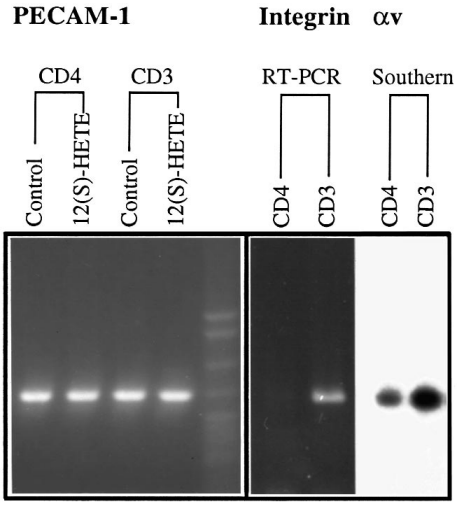


Fig. 12. CD clone 3 (CD3) and CD clone 4 (CD4) endothelial cells express different basal levels of αv mRNA but not of PECAM-1 mRNA. The RT-PCR of PECAM-1 of CD clone 3 and clone 4 cells treated with solvent control or 0.1 μM 12(S)-HETE (4 hours) was performed using primers and cycling conditions as previously described (Tang et al., 1993a). Both endothelial cell lines express similar amounts of basal levels of PECAM-1 message and 12(S)-HETE did not affect the mRNA levels of PECAM-1 in both cell lines. The RT-PCR/Southern blotting of integrin αv was performed as described in the text and for Fig. 1. While CD clone 3 cells express easily detectable amounts of αv mRNA, CD clone 4 cells express very low basal levels of αv message.

4 endothelial cells also is evidenced by northern blotting in which much higher RNA loading and longer exposure time are required to reveal the αv band in CD clone 4 cells than in clone 3 cells (compare Fig. 5 of this study and Fig. 5 of Tang et al., 1994). Another significant difference is that, when stained intracellularly for integrin αvβ3, CD clone 3 cells but not clone 4 cells demonstrate a significant cytosolic pool of this integrin receptor (Fig. 1; also refer to Tang et al., 1993c). Also, αvβ3 in established CD clone 3 cultures is predomiantly localized to focal adhesions (Tang et al., 1993c), whereas the focal adhesion localization of this integrin receptor in CD clone 4 cells is not apparent (Fig. 1). On a theoretical basis, a high basal expression of αv mRNA and the presence of a prominent cytosolic pool of αvβ3 protein in CD clone 3 cells would make it easier for the cells to mobilize the receptors to the surface (from cytosol and/or focal adhesions) via a cytoskeleton-dependent mechanism (Tang et al., 1994). In contrast, the low basal level of the αv mRNA and lack of a 'ready-for-use' cytosolic pool of the proteins makes it necessary for CD clone 4 cells to initiate de novo gene transcription, although the possibility could not be excluded that a post-transcriptional mechanism may also operate in CD clone 4 endothelial cells in response to 12(S)-HETE stimulation.

The molecular mechanism for the specific modulation by 12(S)-HETE of αv gene expression is unclear. The promoters for integrins α2, α5, β1, αL, αM, αX, β2, αIIb, and β3 have been characterized, most of which lack conventional TATA box or CCAAT box but rather have a 5' upstream initiator sequence and consensus binding sites for ubiquitous transcription factors including Sp1, AP-1, and AP-2. Very recently, the promoter for integrin αv has been reported (Donahue et al., 1994). Like most other integrin promoters, the αv gene does not contain a TATA box but does contain four Sp1 binding sites, two Ets binding sites and one GATA binding site. Most of the transcription factors that bind to AP1/AP2/Sp1 have been shown to mediate enhanced transcription via the phorbol ester/diacylglycerol-activated PKC pathway. Previous work demonstrated that phorbol esters upregulated αv gene expression (Suzuki et al., 1987), suggesting the involvement of PKC. 12(S)-HETE has been shown to be a PKC activator (Liu et al., 1991; Tang et al., 1993c, 1994). 12(S)-HETE-induced αv gene transcription is significantly inhibited by a selective PKC inhibitor, i.e. calphostin C, suggesting that the 12(S)-HETE effect on CD clone 4 cell αv integrin depends on PKC activation. As indirect evidence, PKC activator PMA also induced the αv mRNA expression in CD clone 4 cells. In contrast, protein kinase A inhibitor (H8) and protein tyrosine kinase inhibitor (genistein) did not affect 12(S)-HETE induction of αv mRNA. Interestingly, both transcriptional activation of the αv gene in CD4 cells and post-transcriptional translocation of αvβ3 receptor in CD3 cells by 12(S)-HETE depend on PKC activity. It will be of future interest to determine what cell type-specific factor(s) (including *cis*- and *trans*-elements) is responsible for the differential effects of 12(S)-HETE in these two cloned endothelial cell lines and the selective effect of 12(S)-HETE on integrin αv but not other subunits such as α5, β1, and β3.

Enhanced gene transcription of integrin αv in CD clone 4 cells stimulated by 12(S)-HETE results in an increased surface expression of the protein (as demonstrated by both digitized image analysis and flow cytometry) and cell adhesion to vitronectin. Integrin subunit αv can be coupled with β1, β3, or β5 in resting microvacsular endothelial cells (Luscinskas and Lawler, 1994). The exact β-subunit partner(s) of αv in CD clone 4 cells have not been characterized. Flow cytometric analysis demonstrated the surface expression of low levels of both αvβ3 and αvβ5 on the surface of CD clone 4 cells. 12(S)-HETE and PMA stimulation resulted in a significant increase in αvβ3 surface expression (both the percentage of positive cells and mean fluorescence intensity). In contrast, PMA and 12(S)-HETE only marginally upregulated the surface labeling of αvβ5. In contrast to αvβ3 and αvβ5, α5β1 integrin was expressed in significantly higher amount on the surface of CD clone 4 cells. Neither 12(S)-HETE nor PMA demonstrated any effect on α5β1 surface expression, consistent with our molecular biology data. CD clone 4 cells stimulated by 12(S)-HETE or PMA demonstrated an enhanced adhesion to vitronectin, which was inhibited by anti-αvβ3 but not by anti-α5β1. Interestingly, although both 12(S)-HETE and PMA also appeared to upregulate αvβ5 surface expression, anti-αvβ5 (P1F6) did not inhibit the increased cell adhesion, suggesting that this integrin receptor may be involved in some other aspect of cell behavoir such as cell motility.

Integrin αvβ3 is a multifunctional receptor implicated in elevating intracellular pH (Schwartz et al., 1991), inducing Ca^{2+} influxes (Leavesley et al., 1993), signaling collagenase IV gene expression (Seftor et al., 1992), regulating angiogenesis (Brooks et al., 1994) and apoptosis (Bates et al., 1994), and mediating tumor cell adhesion to stimulated endothelial cells (Lafrenie et al., 1992; Tang et al., 1993b, 1994) and matrix proteins (Felding-Habermann and Cheresh, 1993). Thus, increased αvβ3 expression in CD clone 4 microvascular

endothelial cells may have a far-reaching biological impact involving many of the above events mediated by this integrin receptor. 12(S)-HETE, by transcriptionally and/or post-transcriptionally regulating the αvβ3 expression of endothelial cells, may play an important role in many physiological and pathological vascular events such as hemostasis, atherosclerosis, inflammation, angiogenesis, and tumor cell dissemination.

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